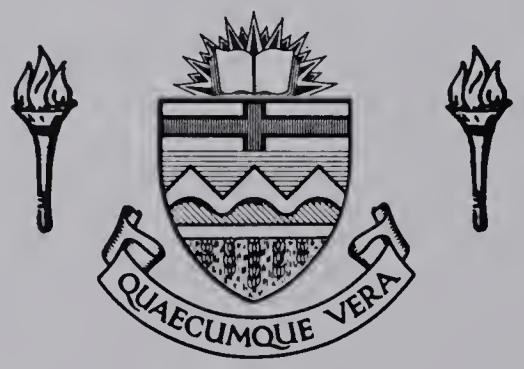

THE UNIVERSITY OF ALBERTA

EFFECTS OF LIGHT, SALTS, AND ETHYLENE ON THE pH OF *EUGLENA* SUSPENSIONS

by

© ENID JOYCE NEWELL

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

FALL, 1971

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled EFFECTS OF LIGHT, SALT, AND ETHYLENE ON THE pH OF EUGLENA SUSPENSIONS, submitted by Enid Joyce Newell, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

This investigation considers light-induced pH changes by both photosynthetic and bleached Euglena gracilis and suggests they might be related to changes in H^+ binding by the cell macromolecules connected with changes in the metabolic state of the cells. The relationship to the CO_2 metabolism of the cell is also discussed, as are pH changes caused by ethylene and salts.

An unbuffered suspension of photosynthetic Euglena gracilis Z increased in pH during the first 5 min that it was exposed to light. The pH returned to the initial value in the dark. A slightly slower, light-induced pH decrease was seen with streptomycin-bleached E. gracilis var bacillaris SM-L1. The total proton movements by photosynthetic cells were increased by 0.03% CO_2, and inhibited by 100 µg/ml Triton X-100. The light-induced pH changes showed an initial 20 sec lag that was not seen in the oxygen evolution by the cells. The suspensions reached a steady pH in the light at a time when duplicate suspensions were showing net O_2 production. The amount of NaOH required to increase the pH of a suspension from 6.0 to 8.0 was greater for cells in the dark than for those in the light.

10 mM concentrations of NaCl and KCl did not affect the pH of suspensions of photosynthetic cells during light-dark cycles, but gave decreases of 0.05 to 0.2 pH when added to suspensions of cells in the dark. Pretreatment with 10 ppm ethylene inhibited this pH change. Ethylene alone increased the pH about 0.04 pH during 15 min application. $MgCl_2$ and $CaCl_2$ (10mM) decreased the pH of both suspensions in light-dark cycles, and of those in constant dark. This effect was not inhibited by ethylene.

These results suggest that the light-induced pH changes are related to changes in proton-binding by the cell macromolecules during the induction phase of photosynthesis. Salts cause release in H^+ from the membrane. Ethylene possibly localizes in the hydrophobic regions of the membrane, causing changes in H^+ binding. These changes may also inhibit the "binding" of Na^+ and K^+, but not the effects of the more tightly bound Ca^{++} and Mg^{++}.

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to my supervisor, Dr. Mary Spencer, for her help and advice in this project. It could not have been done without her encouragement to seek creative ways to approach biochemical problems.

I would also like to thank Bob, my husband, computer programmer, graph plotter, engineering consultant, mathematician, draftsman and general problem solver for his patience with the project.

Thanks also go to all the people who have been part of the research group during the duration of the project for their help and suggestions, and to Drs. C. Atkins and D. Graham of Plant Physiology Division, C. S. I. R. O. Research Laboratories, Ryde, Australia for a helpful discussion.

I would like to thank the Data Acquisition, Control and Simulation Center of the Department of Chemical and Petroleum Engineering for the use of their IBM 1800 computer and Calcomp plotter, and to the Computing Center, University of Alberta, for the use of the IBM 360 Model 67.

Financial support from the National Research Council of Canada, both directly through a Bursary and three Scholarships to the author, and indirectly through a research grant to Dr. M. Spencer, is gratefully acknowledged, as is the award of a Dissertation Fellowship from the University of Alberta.

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INTRODUCTION AND LITERATURE REVIEW

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A. pH Changes in Unbuffered Suspensions of Euglena

Several studies have shown that a suspension of photosynthetic cells in an unbuffered medium shows a pH increase on illumination that is reversed in the dark. These have related the phenomenon to two mechanisms: uptake and release of carbon dioxide by the cells, and uptake and release of H^+ by the chloroplasts.

The former group is most clearly supported by Atkins and Graham (1971) who found a ratio of 1 : 1 : 1 for CO_2 added : O_2 evolved : H^+ uptake by Chlamydomonas reinhardi in the light. Gaffron (1957) and Cummins, Strand, and Vaughan (1969) had previously suggested that CO_2 and bicarbonate were necessary for light-induced H^+ uptake in Chlorella and in Ulva, respectively.

Schuldiner and Ohad (1969) suggested that the light-induced pH changes that they observed in acetate-grown Chlamydomonas reinhardi were related to the uptake and release of H^+ by the chloroplasts. This was not supported by Ben-Amotz and Ginzburg (1969) in their work with Dunaliella parva, a unicellular, halophilic alga.

An interesting corollary to the pH changes in chloroplast suspensions is the light-induced increase in buffer capacity in the light that was noted by Polya and Jagendorf (1969).

B. The Use of Euglena in Studies of Hormonal Action

A second objective of the investigations in this thesis was to use the light-dependent pH changes in suspensions of Euglena gracilis Z as a system to test for effects of the plant hormone, ethylene on the alga. The ability of very low concentrations of such a simple molecule as ethylene to cause gross changes in plants has long interested scientists (see Spencer, 1969), and explanations have been sought through studies on both intact organisms and subcellular components. A unicellular organism permits the study of the interaction of the gas with intact cells. This is of special importance in ethylene research since production of the gas has been linked to wounding of tissues. The gas is produced in small amounts by almost all organisms so far studied, and it is not known if this has a physiological role, or is just a waste product. It was hoped that the current studies would show if a response to low concentrations of ethylene is characteristic of living cells, and might possibly give some idea of the nature of anethylene receptor.

The effects of plant hormones on algae and photosynthetic bacteria have not been studied extensively. The existing studies report mainly changes in cell size and growth rate caused by kinetin, gibberellic acid, and auxins on various organisms (e. g. Romanow, Kaiser, and Pochon, 1969; Ahmad and Winter, 1968).

There are three reports of hormone effects on Euglena. Elliot (1939) reported that 3-indole acetic acid (IAA), indole butyric acid, and indole propionic acid at concentrations of 10^{-7} M stimulated growth of E. gracilis. This effect was not observed in colourless cells, inferring that it might be related to the presence of chlorophyll. Griffin (1957) showed that gibberellic acid at 1000 ppm caused elongation and a possible delay in reproduction and synthesis of chlorophyll in Euglena. Supniewski et al (1957) showed that either kinetin or thiokinetin at 10^{-8} M concentration stimulated the growth of E. gracilis.

C. Possible Mechanisms of Ethylene Action in Biological Systems

Ethylene is physiologically active in plants (e. g. in induction of ripening) at concentrations as low as 0. 1 ppm (Burg and Burg, 1967). This is in contrast to the 80% ethylene needed to attain surgical anaesthesia in man over a much shorter exposure period. It is difficult to say whether the mechanisms of action of the gas are the same in both systems. Because the modes of biological activity of a substance are limited by the properties of the substance, or of a substance that is formed from it, it seems possible that at least some of the basic principles of action of the gas in animal systems would also apply to plants.

Pauling (1962) and later Catchpool (1968), and Miller (1968) proposed a theory of action for ethylene and other non-ionic anaesthetics. They visualized the formation of gas hydrates - clathrate compounds in which the gas is held in a cage formed by water molecules. These structures would block ion movements, and thus induce anaesthesia. Hydrate formation is found in water-ethylene mixtures only at low temperatures or at several atmospheres pressure. It is possible that in biological systems, the ice-like water surrounding hydrophilic regions of proteins would stabilize the hydrates.

Studies on the action of ionic anaesthetics show that they may be able to interact with membranes and alter their strength and permeability (Seeman et al, 1969; Sessa and Weissman, 1967; Johnson and Bangham, 1969).

Possible mechanisms of ethylene action in plants have been reviewed in detail by Spencer (1969). They include formation of hydrates as suggested above, some change in reactivity or arrangement of the membrane resulting from the gas' high solubility in lipids, or activity as a member of an electron transport chain. The last suggestion was

based on the hypothesis that ethylene might be able to donate one of its π electrons to an electron carrier under appropriate conditions, and was advanced as an explanation for the gas' ability to increase the respiratory rate during the climacteric in fruit.

Ethylene has been shown to increase the rate of ATP-and ADP-stimulated swelling in bean mitochondria, and to increase mitochondrial ATPase activity (Olson and Spencer, 1968; Philips, 1971). This suggests a possible role of these sub-cellular organelles as receptors for ethylene.

Burg and Burg (1969) suggested that ethylene might be active as a chelator of metal ions. They have characterized a site of action by defining the necessary molecular characteristics by using analogues, and by considering interactions of the gas with CO_2 and oxygen.

The need for a specific receptor for ethylene action has also been considered by Ku and Leopold (1970). Using equilibrium dialysis, they found that less ethylene was bound by subcellular fractions of "unresponsive" tissues than "responsive" ones, thus inferring the existence of special structures in affected tissues.

Burg and Burg (1969) showed that ethylene was 130 times more active in inhibiting the growth of pea stem sections than propylene, the next most active substance. However, Mehard and Lyons (1970) found no specificity for the action of ethylene over that of similiar gases in changing the rate of KCl-induced swelling of mitochondria, and Mehard, Lyons and Kumamoto (1970) showed a similiar lack of specificity for the activity of the gas in changing the surface tension of artificial membranes of protein and lipids.

In the present work, the light-dependent pH changes in Euglena are characterized and the possible explanations of their origin are considered. This is discussed in relation to salt- and ethylene- effects on

the system.

Two points should be noted.

The first is that the terms pH change, and hydrogen ion movements are used interchangeably although there is no evidence that the movement of other substances is not involved. No distinction is made between the terms: hydrogen ion and proton, and both are used to represent the hydronium ion, H_3^+O, the form that the proton takes in solution.

The second is that the H^+ concentration at a cell-water interface may be 100 times that of the solution (Weiss, 1967). The relationship of the pH changes observed to the actual movements of protons is thus somewhat uncertain.

MATERIALS AND METHODS

A. Sources of Chemicals and Biological Materials

1. Euglena

Euglena gracilis Klebs Z was obtained from the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Indiana (number 793). *E. gracilis* var *bacillaris* SM-L1 (number 945) came from the same source.

2. Chemicals

Chemicals were generally of the highest purity available. Vitamin B_{12} (cyanocobalamin) was from Sigma, and thiamine HCl was from Nutritional Biochemicals. Most other chemicals were from Fisher Scientific Co. Ltd.

3. Gases

Water-pumped compressed air and carbon dioxide used for the aeration of the cultures were obtained from the Canadian Liquid Air Co. Ethylene (C. P. grade) came from the Ohio Chemical Company, Edmonton. Prepared cylinders of approximately 100 ppm ethylene in air were purchased from Matheson and Consumers Welding Supplies, Edmonton.

4. Water

Distilled water from the laboratory lines was passed through 2 cartridges of Barnstead mixed bed ion exchange resin and one of organic removal resin. It was then distilled in a Corning Pyrex still. The resulting water had a conductivity of less than 0.8 ppm NaCl as measured

by a Crystalab Deeminizer apparatus.

B. Basic Culture Conditions

Cultures were grown in a Cel 8 Sherer Gillet (Marshall, Michigan) growth chamber at 21° or 28°. Lighting was provided by six Lifeline Sylvania Fluorescent tubes about 18 inches above the culture. This gave a light intensity of 500 to 640 ft-c at the level of the culture, as measured by a Weston Illumination Meter, Model 756 with a quartz filter.

Cultures were aerated at 50 to 200 ml per min with 1% to 2% CO_2 in air. The mixture was prepared from separate cylinders of compressed air and CO_2 with the aid of two calibrated flowmeters. This was passed through two cotton wool filters. During the later stages of the work, these were supplemented by a 0.22 μ Millipore filter, 25 mm diameter. The cultures were grown in round bottom flasks and stirred continuously by a teflon covered magnet.

The basic culture medium used was the salt medium of Cramer and Myers as modified by Edmunds (1965a): 1.00 g/l $(NH_4)_2HPO_4$, 1.00 g/l KH_2PO_4, 0.20 g/l $MgSO_4$, 0.026 g/l $CaCl_2 \cdot 2H_2O$, 0.516 g/l Na citrate $\cdot 2H_2O$ (chelator), 3.3 mg/l $FeSO_4\ 7H_2O$, 1.55 mg/l $MnSO_4\ H_2O$, 1.3 mg/l $Co(NO_3)_2 \cdot 6H_2O$, 0.40 mg/l $ZnSO_4 \cdot 7H_2O$, 0.24 mg/l $(NH_4)_6 Mo_7O_{24} \cdot H_2O$, 0.02 Mg/l $CuSO_4 \cdot 5H_2O$, 0.1 mg/l vitamin B_1 (thiamine HCl), and 0.5 μg/l vitamin B_{12} (cyanocobalamin). Stock solutions of the above substances were used to prepare the final medium. The $FeSO_4$ was added dry before autoclaving and a filter-sterilized solution (Nalgene Filter Units from Canadian Laboratory Supplies) of the vitamins was added aseptically to the autoclaved medium after cooling.

Autoclaving was for at least 20 min at 15 lb pressure. For large volumes of medium (greater than 2 l), 35 min autoclaving was used.

1. The Basic Culture

(a) Description

The culture apparatus shown in Figure 1 was constructed to permit the continuous culture of organisms for a reproducible source of experimental material. The glassware was purchased from Bellco Glass Co., Vineland, N. J., and the pump from Beckman Instruments Inc. This pump could be easily disassembled for autoclaving and accurately adjusted for pumping rates from 0-2 ml per min. This pump was found to be essential for the practical maintenance of the culture.

The metered streams from the air and CO_2 cylinders were mixed, moistened by bubbling through sterile medium in reservoir D, led through the culture in flask J, and released through cotton filter H. If the exit through H was closed near I, the air was released at O or P depending on the position of stopcock M, thus maintaining a constant fluid level in the culture flask. Because of the slight pressure buildup when the culture was thus being forced into reservoir N, all ground glass joints (all exits on D and J) were routinely secured with joint clamps (Bellco Glass Inc.).

(b) Autoclaving and Assembly

The culture apparatus was completely disassembled for autoclaving. From 2000 to 4000 ml medium were autoclaved in the reservoir, and 750 ml plus the stirring magnet in the culture flask. After the apparatus had cooled and was assembled, the vitamin solutions were injected with a sterile syringe into the reservoir through the inlet tubing, and into the culture flask through the injection port G. The inoculum was prepared by washing the surface of an agar culture with sterile medium. This was also injected through port G.

Figure 1. The basic culture apparatus.

A. In flow meter

B. Cotton wool filter

C. Millipore filter holder with filter (0. 22μ)

D. Medium reservoir (4 l)

E. Medium addition port

F. Medium pump (Beckman solution metering pump)

G. Injection port for inoculation of culture

H. Cotton wool filter on air outflow (often supplemented with a Millipore filter, I)

J. Culture flask (1 l) (In addition to the three outlets shown, a fourth permitted rapid removal of almost the total culture volume)

K. Magnetic stirrer

L. Large capillary culture withdrawl tube

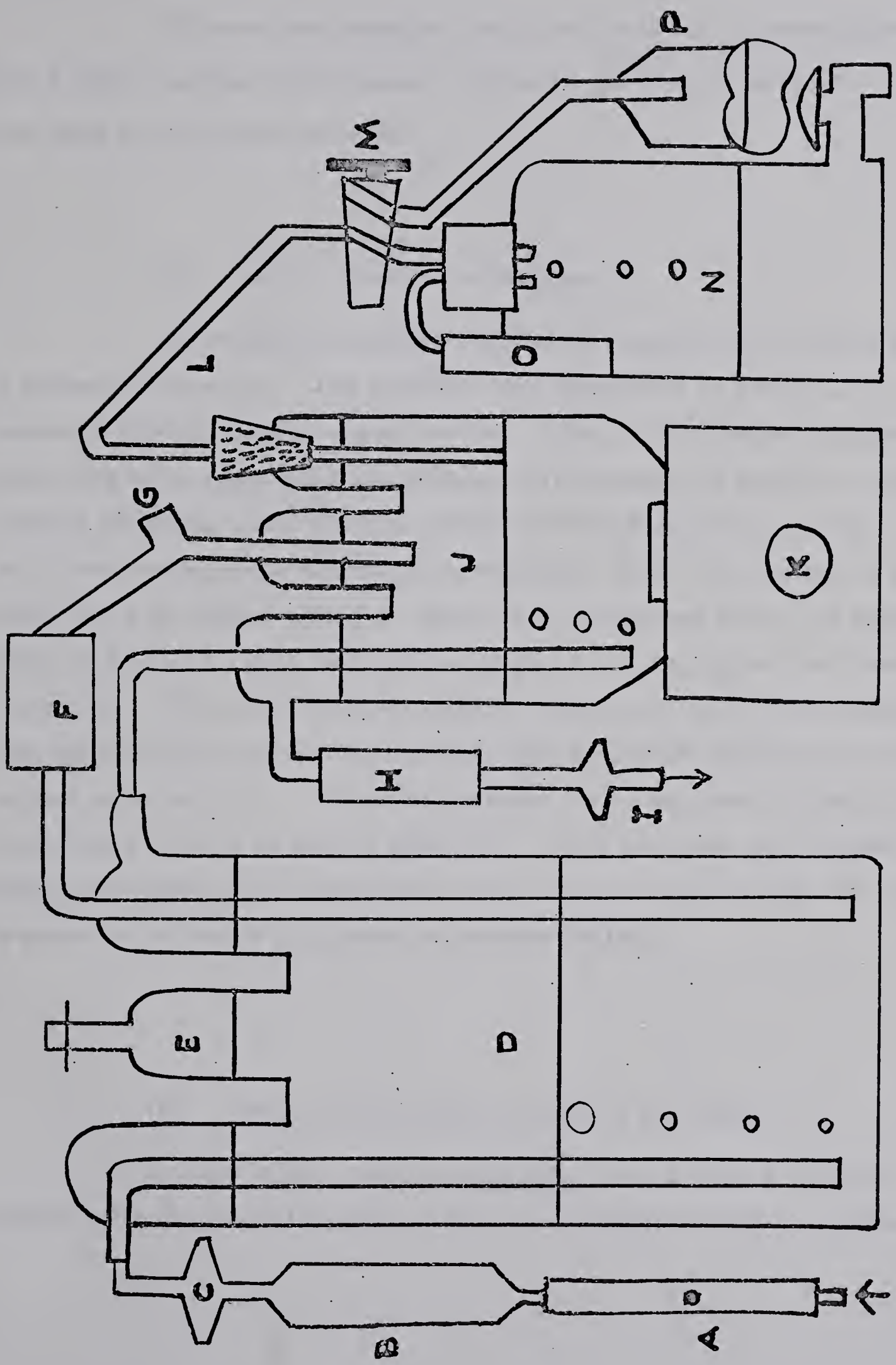
M. 3 - way stopcock

N. Culture overflow reservoir

O. Cotton wool filter

P. Culture sampling outlet with aseptic filling bell

approximate scale 1/3 in = 1 in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Cultures were examined daily for bacterial contamination with a phase contrast microscope. Only cultures free of bacteria were used for experimental work.

(c) Aseptic Addition of Medium

The method of aseptic addition of medium to the reservoir is shown in Figure 2. The medium was autoclaved in bottle C, a standard 2400 ml glass reagent bottle. After it had cooled, stopper B, which had been autoclaved separately, was put into the bottle, and the vitamin solutions were added aseptically through injection port D. Outlet A was connected to the main air manifold, and E was joined to the short piece of rubber tubing F. which was autoclaved on G, the medium inlet, and closed with a short piece of glass rod during normal culture operation. When the air to the culture was closed off, it was diverted into the medium transferring system, and forced the medium into the culture reservoir H. When the transfer was completed, tubing F was closed with a piece of sterile glass rod. The transfer was stopped before the bottle was completely empty and air swept through the system in order to decrease the chance of contamination.

(d) Measurement of the Growth of the Culture

Growth of the culture of Euglena was followed by direct counts of formalin-killed cells with a 1 ml Sedgwick-Rafter counting

Figure 2. Aseptic addition of medium to culture reservoir.

A. Outlet for connection to air line

B. Rubber stopper

C. Glass reagent bottle

D. Injection port

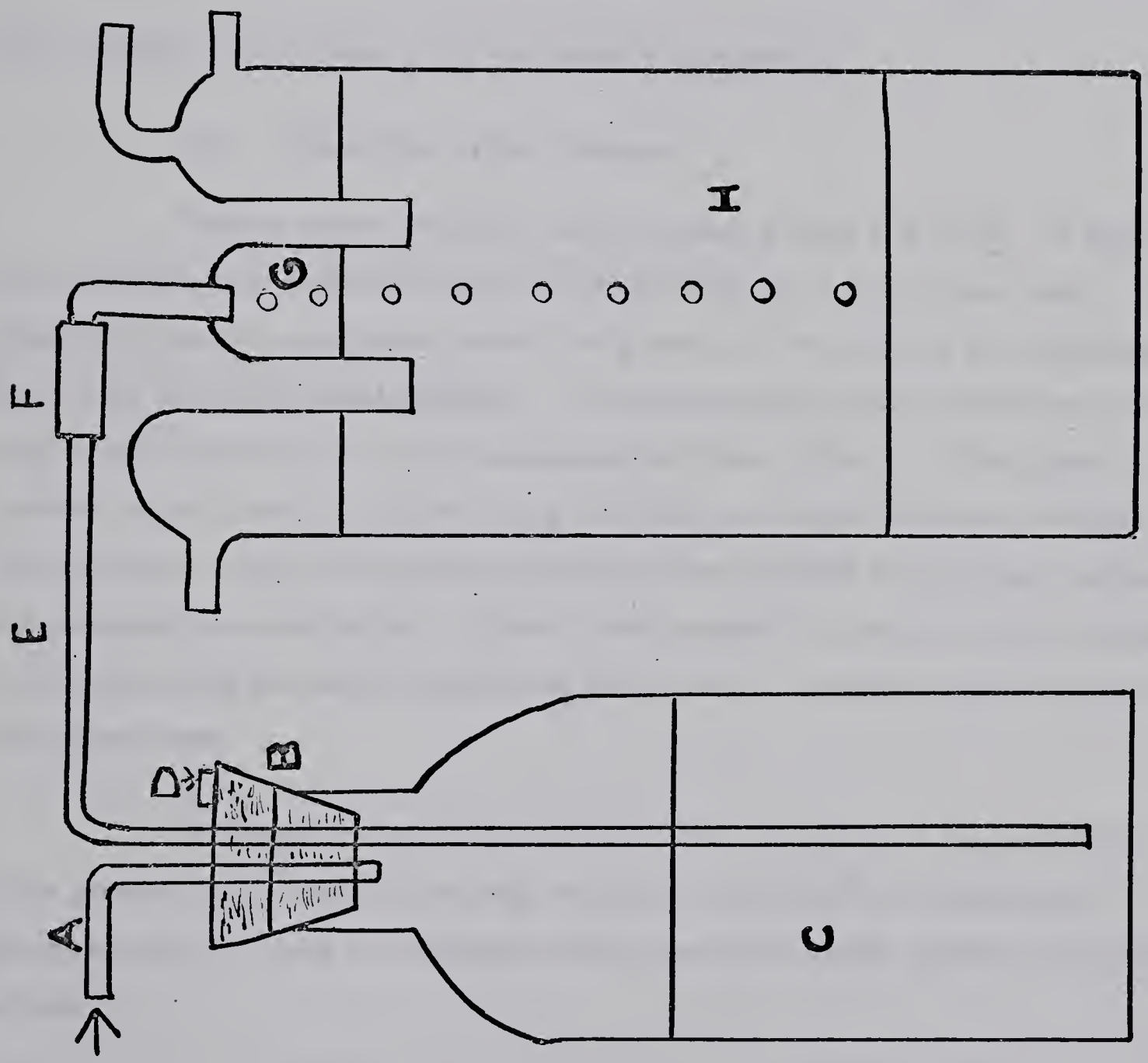
E. Medium transfer tube

F. Rubber tubing

G. Medium inlet

H. Culture reservoir

approximate scale 1/3 in = 1 in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chamber and a Wild M20 phase contrast microscope at 156 X magnification. The diameter of the microscope field was measured using the grid of a Levy counting chamber. The ratio of the total area of the counting chamber to the total area of the microscope field was used in determining the number of cells per ml. The number of cells in 5 or 10 fields (from 200 to 700 cells) was determined, and the cell concentration was calculated from the formula: Number of cells/ml = $\frac{\text{number of cells/n fields}}{n} \times 765$

This method gives about a 5% variability in results.

(e) Operation of the Culture

Synchronized cultures were grown under a L D:14, 10 light cycle and diluted, only during the light period, at 0.73 ml per min. This suggested that the conditions were too good for the cells to be sufficiently limited by the controlled lighting. The temperature was therefore lowered to 21° close to the conditions used by Cook (1961). This gave division rates about 0.9 divisions per cycle, an improvement over the previous rate since less growing medium was needed to keep the culture at a constant concentration. Under both conditions, most of the divisions occurred during the dark period, so there was a certain degree of synchrony present.

Chlorophyll was determined by the method of Arnon (1949). Cells grown at 21° had an average volume of 2750 μ^3 and contained approximately 14 $\mu\mu$g chlorophyll when measured at the middle of the light period.

2. Stock Cultures

Stock cultures were grown on nutrient agar (Fisher Scientific Co.) in either room light or in the growth chamber, and were recultured monthly. At first, liquid stock cultures were also kept with Na acetate

as a carbon source. These seemed prone to contamination and were discontinued.

3. Euglena gracilis var bacillaris SM-L1

E. gracilis var bacillaris SM-L1 was grown in stirred cultures in same medium with 0.05 M Na acetate as a carbon source. The pH was 6.8. The cultures were maintained at 21° or 28° and aerated with either CO_2-free air or 1% CO_2 in air. The cultures were usually covered with aluminium foil to block out most of the light of the growth chamber.

C. pH Changes in Unbuffered Cell Suspensions

1. The Washing of the Cells

Glass-distilled water was used to wash and suspend the cells. It was boiled in a 500 ml Erlenmeyer flask to remove dissolved CO_2 (Vogel, 1962, p-172), and the flask was immediately stoppered with a two-hole silicone rubber stopper. One hole of the stopper was closed with a tube of Lithasorb CO_2 absorbent, while the other held a tube for removing water from the flask. The water was transferred by vacuum to the separatory funnel shown in Figure 3.

Fifteen ml of the culture were washed 3 times with 15 ml CO_2-free water using a 25 mm Millipore filter holder fitted with a 0.8 μ Millipore filter as shown in Figure 3. For each washing of the cells, the volume of the suspension in the filter holder was lowered to about 5 ml, the stopper B was used to close the top of the holder, and the remaining liquid was sucked off. The vacuum was released as soon as the filter was almost dry, and the resulting backpressure into the filter holder forced the cells off the filter so they were well suspended by the next aliquot of wash water. This technique both ensured thorough washing of the

Figure 3. Apparatus for filtering and washing cells.

A. Separatory funnel (500 ml)

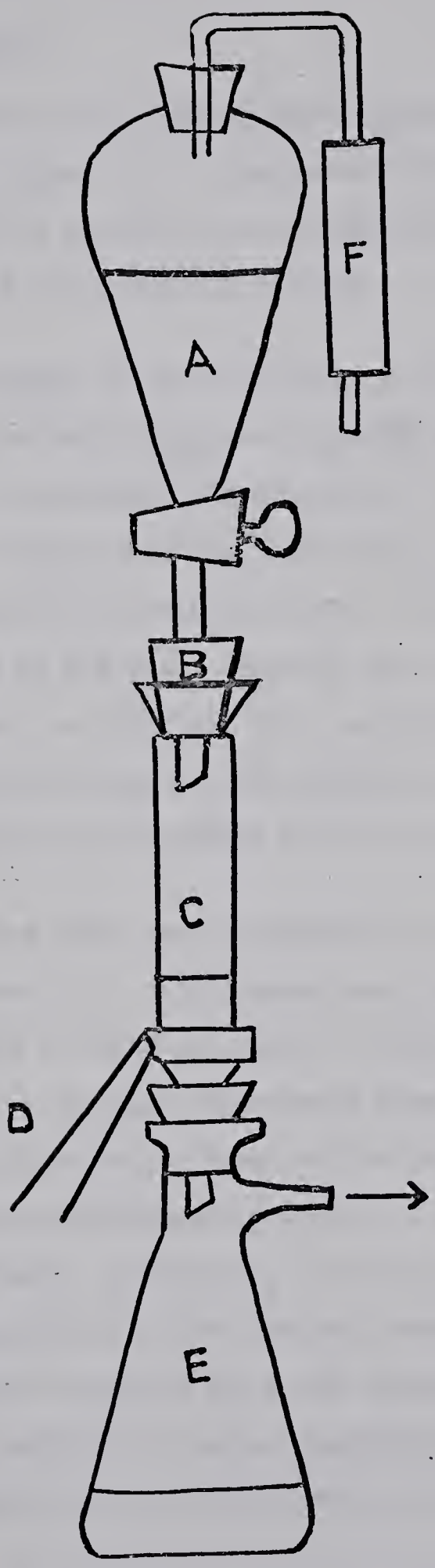
B. Rubber stopper

C. Millipore filter holder (15 ml - 25 cm filters)

D. Clamp for filter holder

E. Vacuum flask (250 ml)

F. Lithasorb filter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cells, and enabled the maintenance of relatively CO_2-free conditions. The cells were suspended in 3 ml water after the final washing.

2. pH Measurements

Light-dependent pH changes by Euglena were measured in the apparatus shown in Figure 4. A Radiometer TTT1 titrator was used as a pH meter, and the output was recorded on a Sargent MR recorder (25 volt setting - 2.5 pH units full scale).

Most suspensions were aerated at 3 or 25 ml per min through a Pasteur pipette to maintain CO_2-free conditions. Carbon dioxide-free air from a cylinder was bubbled through 0.1 N H_2SO_4, then passed through a tube of silica gel before entering the sample. The former treatment removed basic impurities, while the latter removed any H_2SO_4 that may have been picked up by the air. Except when the sample was being treated with ethylene, an ethylene trap consisting of a U tube holding about 50 g silica gel that was soaked with mercuric perchlorate and held in ice water was also included in the air stream.

The suspended cells were injected by syringe into the 15 ml water-jacketed vessel, E. The vessel was darkened with an opaque black cloth, and stirred by a small magnetic stirrer. The light used was a 75 watt General Electric spotlight supported about 5 inches from the culture. The intensity of the light at the level of the culture was 1600 ft-c as measured by a Weston Illumination Meter. A 1 inch plexiglass filter, B, containing constantly circulating ice water was used as a heat shield The change in temperature of the sample when the light was turned on was less than 0.5°. The intensity used was saturating for the light-induced pH changes. The addition of copper sulphate to the water in the filter to block infrared radiation did not affect the response of the cells to light.

The initial pH of the suspension was between 6.1 and 7.0 and was sometimes adjusted using 10 µl Hamilton syringes and 0.05 N solutions

Figure 4. Apparatus for measuring light-induced pH changes in Euglena.

A. 75 watt General Electric Spotlight

B. Plexiglass filter. Arrows show direction of flow of ice-cold water.

C. Wratten filter, when used

D. Radiometer TTA-3 Titration Assembly vessel support with magnetic stirrer

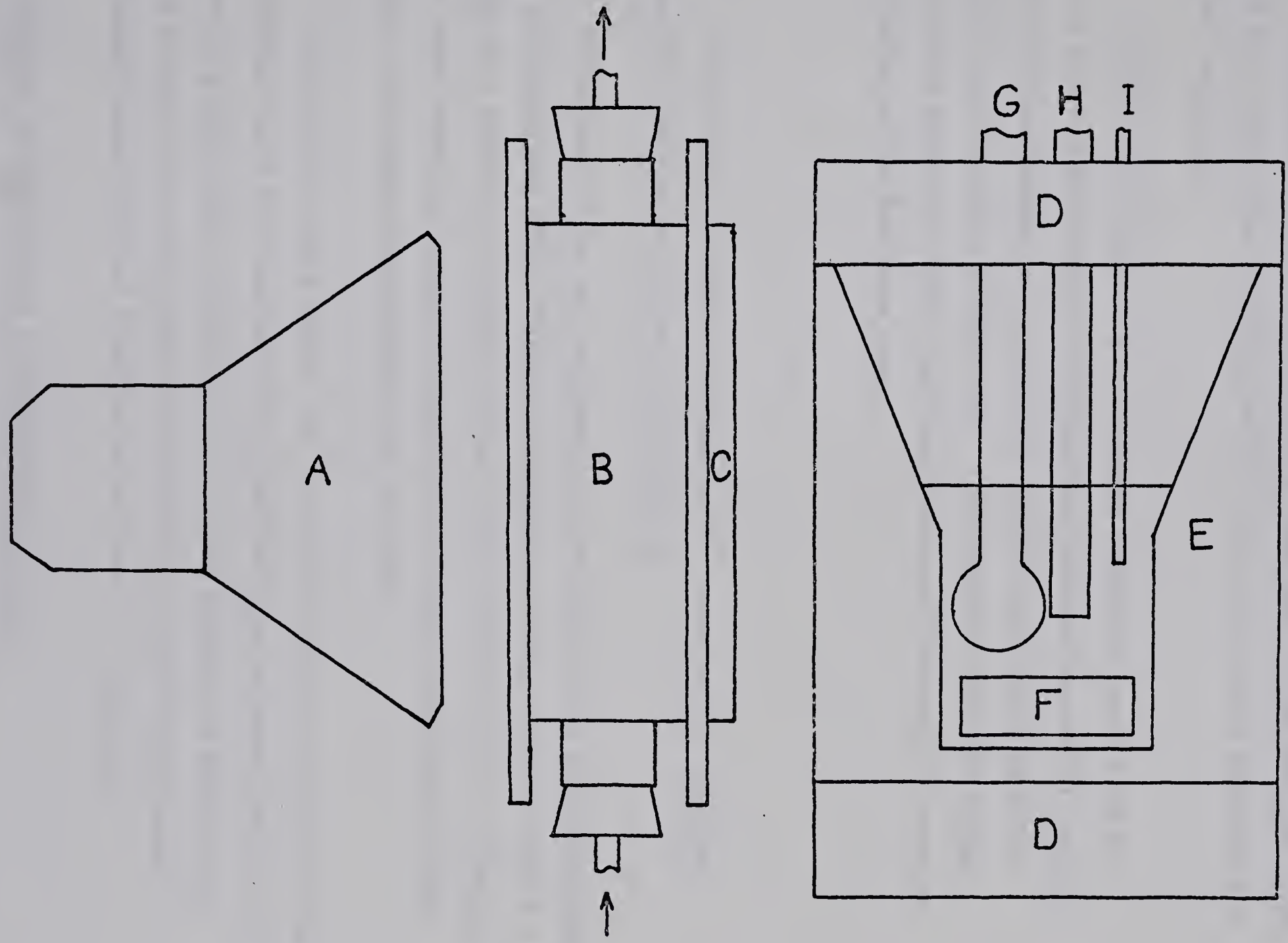
E. Water jacketed reaction vessel

F. Teflon-coated stirring magnet

G. Radiometer G-222C semi-micro glass electrode

H. Radiometer semi-micro calomel electrode

I. Pasteur pipette for aeration of the sample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of NaOH or HCl. Care was taken to avoid bumping the electrodes against the reaction vessel and to check for uniform stirring in order to obtain reproducible readings.

Additions of salts, concentrated solutions of ethylene and Triton X-100 were made with a syringe with the tip of the needle immersed in the cell suspension.

Light-dark cycles of 10, 7.5, and 5 minutes were used at various parts of the study. The light was switched on and off with an automatic time switch. The shortest cycles often barely gave the sample time to reach a steady pH. Calculations from the longer cycles show that the results would have been qualitatively the same if the shorter period had been used.

Calculations of total movements of H^+ occuring during light-induced pH changes are shown in Appendix A.

Titrations of the cell suspensions were done in the above apparatus using a Radiometer SBR2 Titrigraph and SBU1 Syringe Burette with a 1 ml syringe as a source of constant flow of 0.003 N NaOH titrant. Titration values were routinely estimated from the pH change resulting from the addition of an aliquot of standard 0.01 N NaOH.

The titration index (equiv OH^- used/unit pH) was calculated from the titration curve by a computer program that approximated the slope of a curve at a given point as the slope of the line joining two points equi-distant to each side of the given point (method of central differences). Best results were obtained if points were chosen 0.1 pH apart.

D. Changes in Absorbance of Euglena Suspensions

Changes in absorbance were measured with a Cary 15 recording

spectrophotometer at 500 nm. The cells were washed as for the pH measurements, and stirred rapidly with a small battery-operated motor with a plastic stirring shaft. Additions to the suspensions were made with a length of small gauge plastic tubing attached to a syringe.

E. Cell Volume Studies

Microscopic measurements were made on formaldehyde-killed cells with a Wild M20 phase contrast microscope at 625 X using a Whipple ocular micrometer that was calibrated against the markings on a Levy counting chamber. Actual volumes in cubic microns were calculated from the length and width measurements using the formula:

$$\text{volume} = \frac{1}{6} \times \text{length} \times \text{width}^2 \times 2.93$$

where 2.93 is a correction factor to convert micrometer units into microns. The formula determines the volume of a prolate spheroid, the volume defined by the rotation of an ellipse around its major axis. This gives only a rough approximation of the volume of the cells since the majority of cells only approximate the above shape (Corbett, 1957). The volumes, the mean volume of the 18 cells measured per sample, and the significance of the variations of these means and their standard deviations (Student's t test and F test) were calculated using a computer program run on an IBM 360/67 computer.

All measurements were made within 10 min of killing the cells. No significant swelling was observed during this period.

F. Ethylene Analysis

1. Ethylene Collections

Ethylene was collected from the stream aerating a culture by a method similar to that used by Stinson and Spencer (1969).

The compressed breathing air-CO_2 mixture was freed of ethylene by passing it through a U tube (1.4 cm ID) containing about 50 g silica gel (28-200 mesh) coated with mercuric perchlorate and maintained at ice-water temperatures. This achieved a background level of 0.03 nl C_2H_4 per litre air.

After passing through the culture, the air was dried with Drierite, and the CO_2 was removed with Lithasorb. Then the air went through a cold trap and the collection tube, both which were held in a Dry-Ice acetone bath. The collection tube (3.5 mm ID U tube) contained 0.5 g silica gel (Davison, grade 15, 35-60 mesh - Fisher Scientific Co.).

Immediately before collecting ethylene, the collection tube was placed in boiling water for at least 15 min while flushing with purified air to remove any residual ethylene. After the collection, the tubes were sealed with rubber tubing and stored in a Dry-Ice acetone bath until gas chromatography measurements were made.

2. Ethylene Analysis

For analysis, the U tube, still held in Dry-Ice acetone was connected to a two-way valve inserted into the helium line of a Perkin Elmer Model 811 flame ionization gas chromatograph. The valve was opened, and the tube was flushed with helium for about one minute. The valve was then closed and the U tube was heated to 40° to release the ethylene. On opening the valve, the contents of the tube were flushed onto a 50 cm x 6 mm ID column that contained activated alumina coated with 2.5% silicone 550. The column temperature was 20° and the detector temperature was 125°.

A standard curve was obtained by injection of known amounts of ethylene by a gas tight syringe into the gas chromatograph and plotting

the peak height vs nl ethylene. A typical curve is found in Appendix B.

G. Photosynthetic Oxygen Evolution and Respiratory Oxygen Uptake

Oxygen uptake and evolution was measured at 28.0° or 21.0° with a Model 53 Oxygen Monitor equipped with a Clark fixed voltage polarographic probe (Clark, 1956) (Yellow Springs Instrument Company, Yellow Springs, N.J.). A Beckman 100 mV potentiometric recorder (chart speed 0.24 in/min) provided a continuous tracing of oxygen concentration with time.

Respiration and photosynthesis by stirred suspensions of cells were followed in consecutive 5 or 10 min dark and light intervals. Light was provided by a 75 watt GE reflector spotlamp 5 inches from the sample. Heat from the light was absorbed by a 1 inch thick plexiglass filter through which ice water was continuously circulated, and by the thermostated water bath of the oxygen monitor. For the dark period, the probe and water bath assembly were carefully covered with an opaque black cloth.

In later work, when an automatic time switch was used to turn the light on and off, the black cloth was placed covering the whole apparatus except the cold water filter. There was no detectable oxygen release by the cells under these conditions when the light was off. The calculations to determine oxygen evolution and uptake are shown in Appendix C.

H. Salt-induced pH Changes

Unbuffered 1.5 M solutions of various salts were used to test the effect of salts on suspensions of Euglena. The pH's of these solutions were as follows: NaCl - 6.00, KCl - 5.25, K_2SO_4 - 6.40, $MgCl_2$ - 5.70, $MgSO_4$ - 7.05, $CaCl_2$ - 5.95.

RESULTS

A. Characteristics of the Response of the pH Measuring System

Figure 5 compares the time course of the response of the pH measuring system to additions of base to deionized and to buffered solutions with the pH response of a suspension of Euglena in distilled water to light. The much slower response in deionized water is related to the slow response characteristics of glass electrodes at low ion concentrations (Britton, 1955). The pH response of Euglena was of a distinctly different time course, suggesting that there was no sudden uptake of H^+ by the cells. The pH increase of the cell suspension stopped almost immediately on turning off the light.

The pH meter did not respond when the actinic light was turned on, or to the aeration of CO_2-free distilled water with CO_2-free air.

The sample was exposed to a small, slowly increasing amount of KCl that leaked from the reference electrode. This was roughly equal to 5 µl saturated KCl per hour or 1.3 mM KCl in 10 minutes in a 3 ml sample.

B. pH Changes in Suspensions of Photosynthetic E. gracilis Z.

1. General Characteristics

Figure 6 shows the response of a suspension of photosynthetic Euglena in distilled water to alternating light-dark cycles.

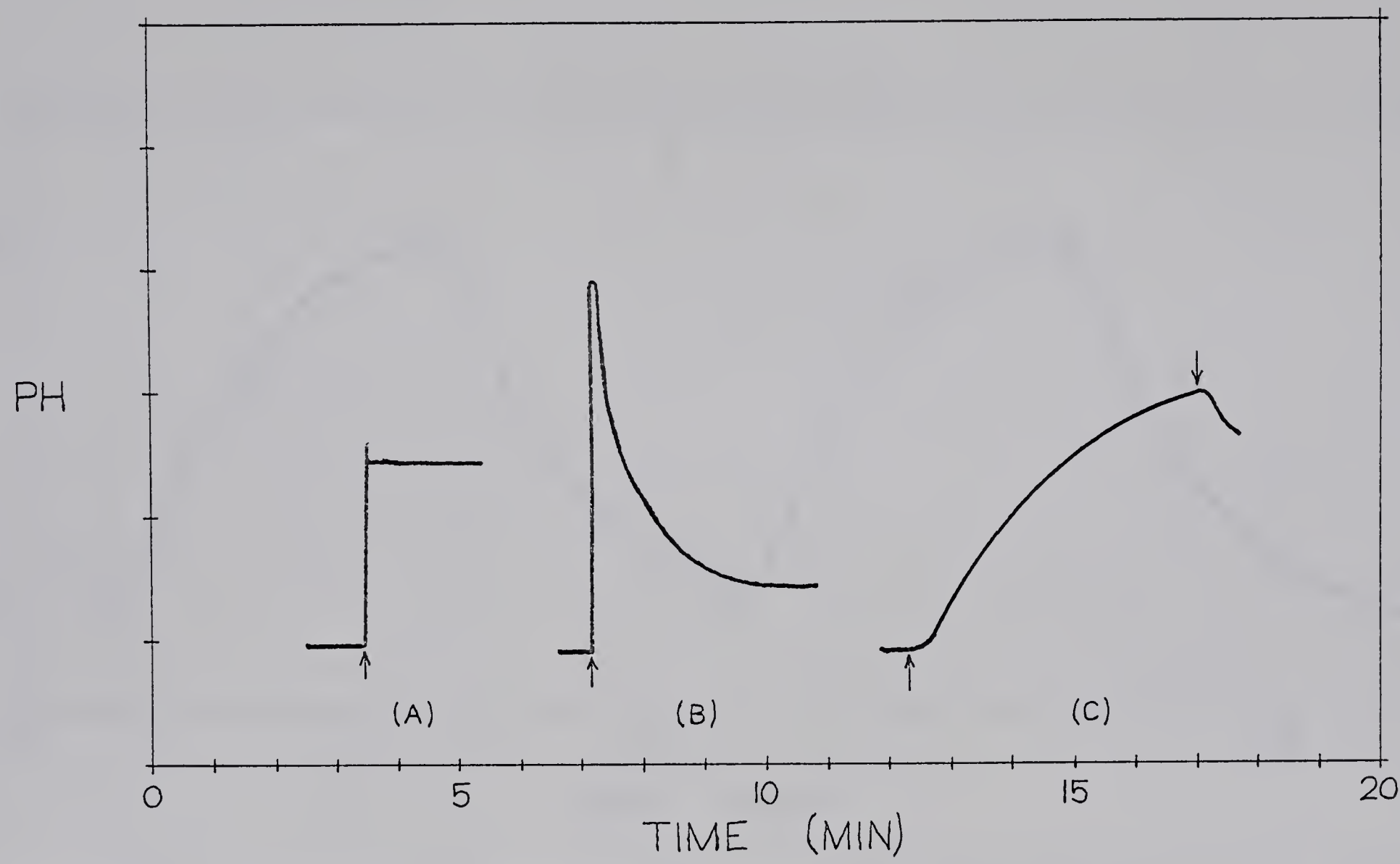


Figure 5. Response of pH recording system to various manipulations. (A) Addition of NaOH to 1.0 M phosphate buffer. (B) Addition of NaOH to CO_2-free distilled water or to a suspension of Euglena in CO_2-free distilled water. (C) Effect of light on pH of a suspension of Euglena in CO_2-free distilled water. The arrows in (A) and (B) indicate the time of addition of NaOH. In (C), the upright arrow shows the time of turning on the light, and the inverted arrow indicates that the light is turned off. Each division on the ordinate represents 0.1 pH units.

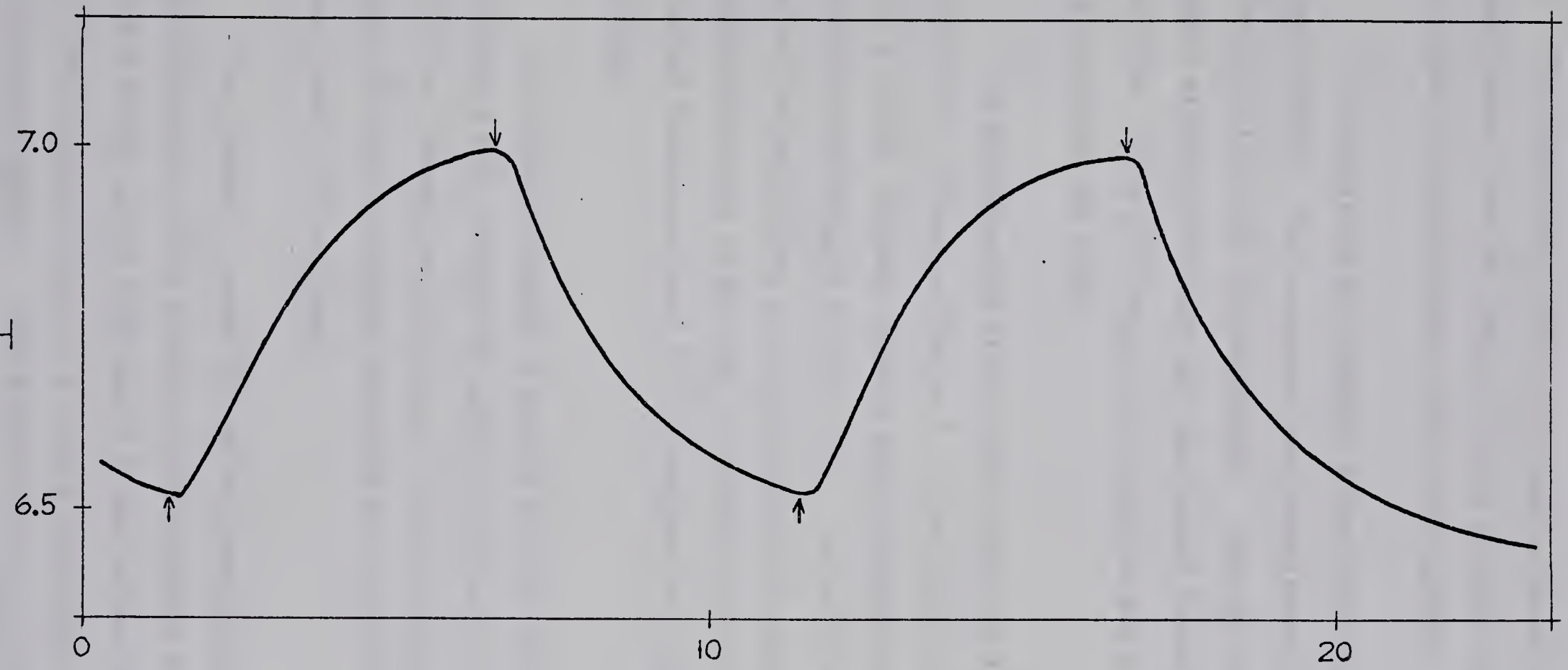


Figure 6. pH changes in a suspension of Euglena in distilled water and exposed to light-dark cycles. The suspension (3 ml of 5.3 x 10^5 cells/ml) was aerated at 3 ml/min with CO_2-free air. (↑) light on ; (↓) light off

The pH change in light or dark started after a lag time of about 20 seconds. The final pH of either the light or dark cycle was stable for several hours, and the final pH value of consecutive cycles was constant within 0.02 pH in about 70% of 64 samples.

The observed pH changes were physiological response of Euglena to light. The response was completely eliminated by killing the cells in 4 mM formaldehyde. The pH change when the temperature of the suspension was decreased from 28^{o} to 21^{o} was less than 0.04. The pH changes therefore do not result from temperature changes by the light.

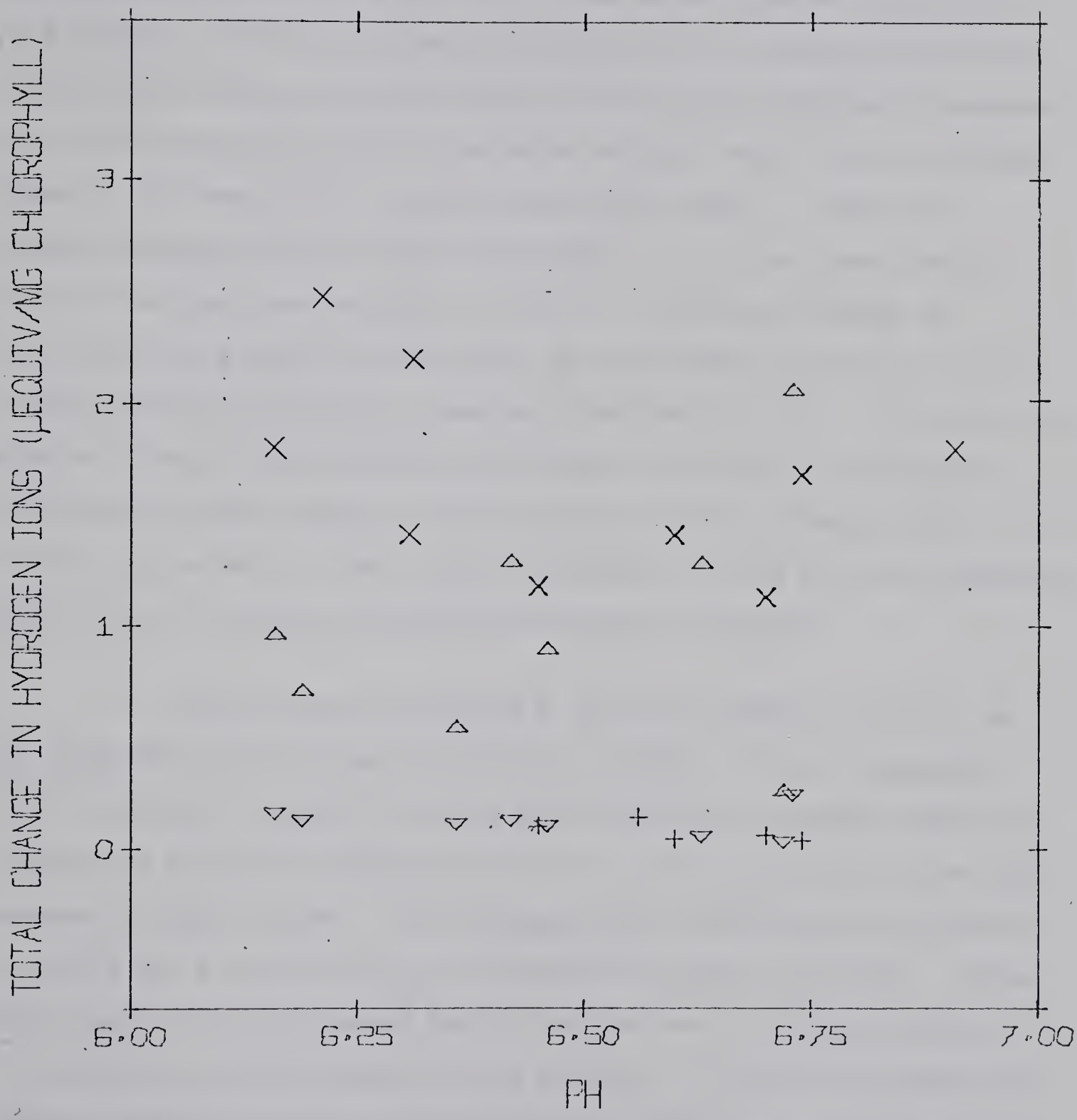
The relationship of the total extent of H^{+} movements to the external pH is shown in Figure 7. The observed pH changes represent 1 to 10% of these values; the remainder result from the buffering of the pH changes by the cells. As will be shown later, at least part of the variability of the extent for the same pH may result from small differences in the CO_2 concentrations of the suspensions. The somewhat decreased total H^{+} movements with high aeration support this view.

The extent of about 2 μequiv H^{+}/mg chlorophyll in the absence of added CO_2 compares with 9 to 10 μequiv H^{+}/mg chlorophyll reported by Ben-Amotz and Ginzburg (1969) for Dunaliella parva and 0.65 μequiv H^{+}/mg chlorophyll reported by Jagendorf and Uribe (1966) for broken spinach chloroplasts.

The times to reach 75% of the total change in pH, and in proton concentration if this occurs simultaneously with the pH change, were about 2.0 min in the light and 3.0 min in the dark. Schuldiner and Ohad (1969) showed times of 1.5 min in the light and 2.8 min in the dark in Chlamydomonas. The figures for Dunaliella parva (Ben-Amotz and Ginzburg, 1969) are 2 min light and 7.5 min dark. pH changes by

Figure 7. Variations in light-dependent H^+ movements in Euglena with external pH. Suspensions of Euglena in distilled water (3 ml of 1.7 to 3.8 x 10^5 cells/ml) were exposed to 5 min light-dark cycles. Titration capacity of the suspension was calculated from the pH change following the addition of a known amount of NaOH (usually about 0.02 μmoles) to the suspension in the dark. The H^+ movements shown by a pH decrease in the dark are plotted against the steady pH in the dark. Each point represents a different suspension of cells. Sample calculations are shown in Appendix A.

	Total increase in H^+ in solution	Change in H^+ from pH change only
Low aeration (3ml/min)	X	+
High aeration (25ml/min)	△	▽


TOTAL CHANGE IN HYDROGEN IONS (UEQUIV/MG CHLOROPHYLL)
2
1
0
6.00
6.25
6.50
6.75
7.00
PH

chloroplasts are more rapid, reaching a maximum change in less than 1 min (Jagendorf and Uribe, 1966).

The accurate estimation of the extent of the reaction is complicated by the change in titration capacity in the light, and in the dark, as is shown in Figure 8. (The term titration capacity is used instead of buffering capacity because it is not known what proportion of the measured value results from actual buffering phenomena (i. e. acid-base interactions), and what amount comes from unrelated uptake or release of H^+ or other ions by the cells). Since the titration capacity of the cells in the light is much less than that for those in the dark between pH 6. 0 and 7. 5, and the pH change is practically the same as in the dark, the calculated extent for cells in the light would be much less than for those in the dark. The observed balance between light and dark pH changes could only be attained if the titration values changed synchronously with the change in pH. It is thus impossible to make a good estimation of total proton movements when the rate of change of titration capacity is unknown.

The two parts of Figure 8 show two different aspects of this light-induced decrease in titration capacity. Part A suggests that the titration capacity in either light or dark is constant with time, because the difference between the dark curves is much less than that between the light curves. This suggests that the difference between the two is not a result of CO_2 accumulation by the suspension. The slight increase in the second dark titration over the first at pH 6. 5 may indicate a small amount of CO_2 build-up. Part B shows that the titration capacity returns to approximately the previous dark values after a light cycle.

Titration of the supernatant fraction after removing the cells by filtration gave a titration capacity of about 0. 05 μequiv OH^-

Figure 8. Titration capacities of suspensions of Euglena in distilled water. The units of the titration capacity are μequiv OH^- used/pH per mg chlorophyll, except for the water sample, which is in μequiv OH^- used/pH per ml. Set A shows the titration of 5 ml of 1.4×10^5 cells/ml suspension with 0.003 N NaOH. Set B was 3 ml of 2.7×10^5 cells/ml suspension titrated with 0.006 N NaOH. Each titration took about 10 min.

Set A

▷ 5 ml water used to suspend cells

▽ Dark for 12 min before titration

◁ Dark titrated immediately after previous titration

\+ Light for 11 min before titration

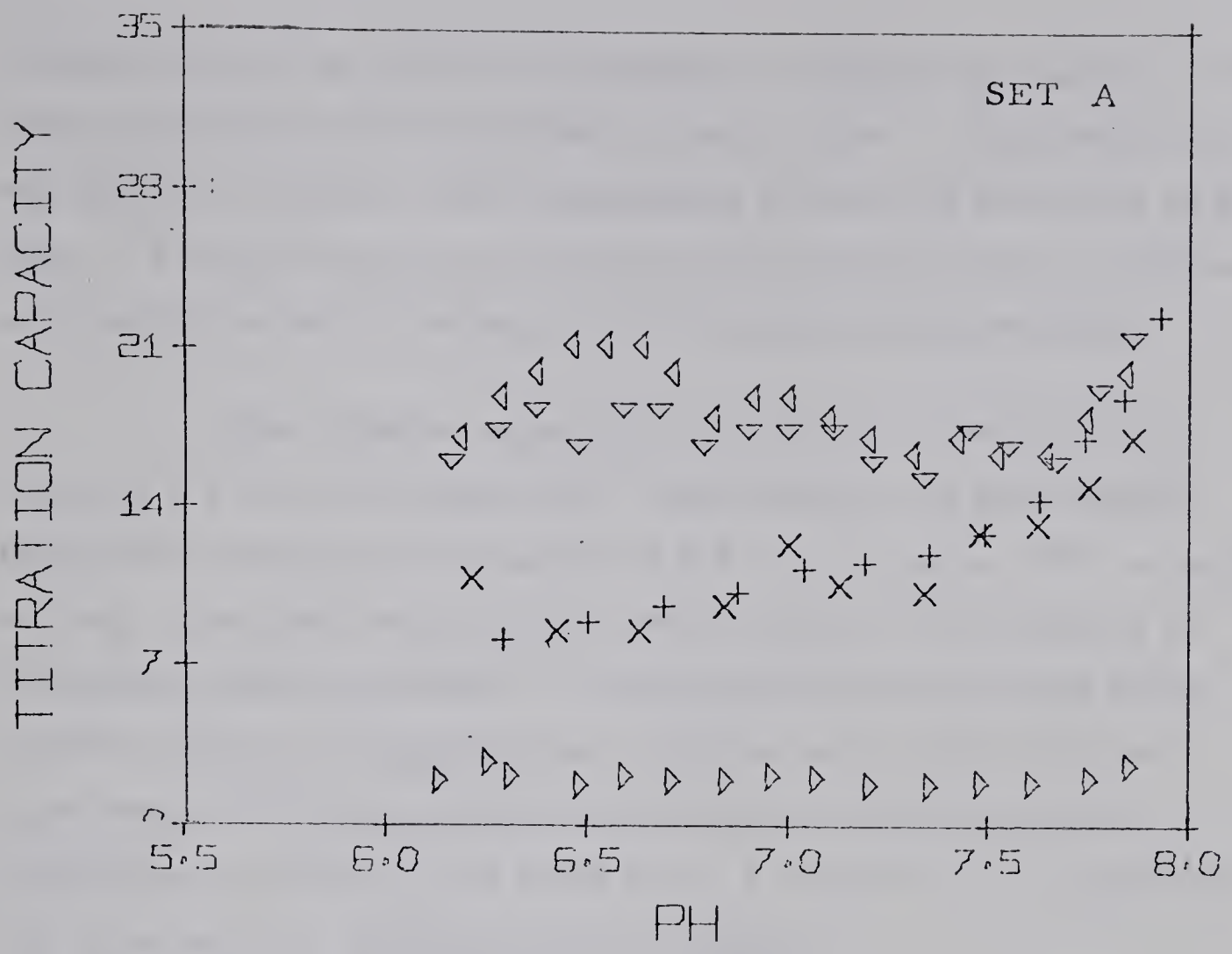
X Light for 60 min after previous titration

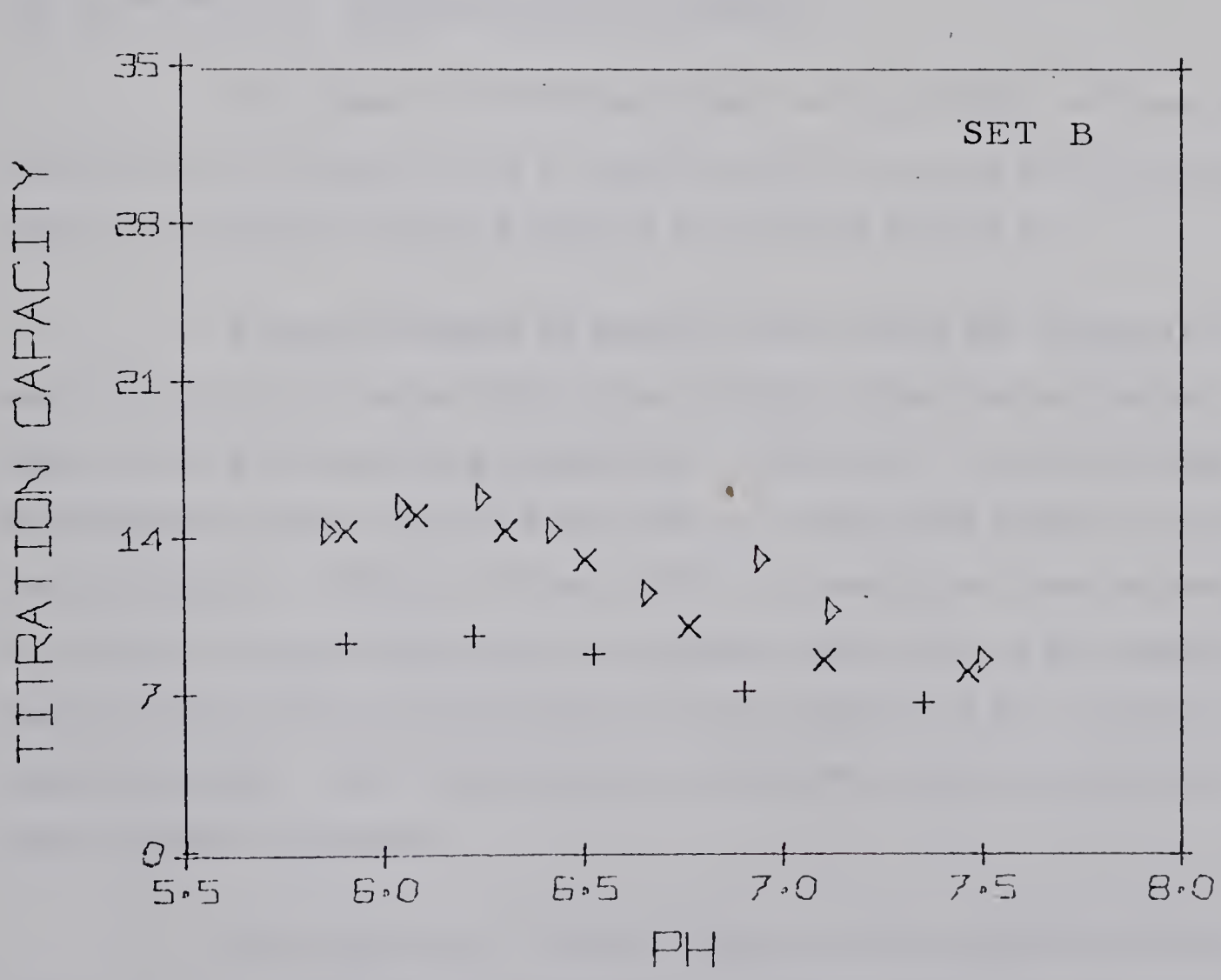
Set B

X Dark for 15 min before titration

\+ Light for 20 min before titration

▷ Dark for 15 min before titration


SET A
TITRATION CAPACITY
35
28
21
14
7
0
5·5
6·0
6·5
7·0
7·5
8·0
PH


SET B
TITRATION CAPACITY
35
28
21
14
7
0
5·5
6·0
6·5
7·0
7·5
8·0
PH

used/pH unit per ml. This is equivalent to roughly one quarter of the total suspension, and about twice that of water alone. This value was the same for samples from suspensions in the dark and those in the light. The pH changes are therefore not caused by the secretion of some acidic or basic substance into the supernatant fraction.

The titration capacity for unaerated suspensions of between 7.0 and 21.0 μequiv OH^- used/pH per mg chlorophyll (Figure 8) compares with values of 2.0 to 4.8 μequiv OH^- used/pH per mg chlorophyll reported for swollen spinach chloroplasts by Polya and Jagendorf (1969). The light-induced decrease of the titration capacity in Euglena was opposite to the light-induced increase in titration capacity observed by the above workers. Schuldiner and Ohad (1969) found about 6.0 μequiv OH^- used/pH per mg chlorophyll in Chlamydomonas reinhardi.

The change in titration values between light and dark was qualitatively reproducible in 6 determinations, as was the decreased difference between light and dark at pH greater than 7.5.

A rough analysis of the spectrum of the pH response was made by placing Wratten filters immediately after the cold water filter in the pH measuring apparatus. Filter No. 25, transmitting wavelengths greater than 570 nm, did not change the extent or rate of the pH change. Filters 65A and 47B, with maximum transmittances at 500 and 430 nm respectively, both gave about 25% of the total pH change when a 300 watt spotlamp was used instead of the 75 watt one normally used. This dependency on red light is characteristic of photosynthetic systems.

The magnitude of the pH change varied with the intensity of the actinic light. One hundred ft-c gave an initial rate about one third that found at 250 ft-c, 1600 ft-c, or 8000 ft-c. The extent of the reaction at the lowest intensity was about one third of the maximum

for the first 5 min, and the pH increase continued for at least 7 min more. These measurements showed that 1600 ft-c was adequate to achieve the maximum pH changes from the cell suspensions.

There was no reproducible difference in the extent of H^+ movements between samples of cells taken at different times from the synchronized culture. The degree of synchrony attained may not have been large enough to show any age-related differences that might exist.

2. Effects of Carbon Dioxide on the Light-Induced pH Changes

The effects of CO_2 on the light-induced pH changes in Euglena were tested by changing the aeration of the sample from CO_2-free air to CO_2 at a known concentration in air. The results are summarized in Table 1. The gas greatly stimulated the H^+ movements, mainly by increasing the titration capacity of the suspensions.

The effect of this addition of CO_2 on the respiratory oxygen uptake and the photosynthetic oxygen evolution was determined in similarly treated suspensions of cells in the closed chamber of a Clark electrode. Tracings from the polarographic curves are shown in Figure 9, and compared with tracings from pH recordings for the same samples of cells. The rate of respiratory oxygen uptake by the cells was not changed by the addition of CO_2.

Photosynthetic oxygen uptake continued for more than 5 min in all suspensions, especially those treated with CO_2. Although it was difficult to make a good comparison between the samples measured for pH changes and those measured for oxygen production because separate equipment was used, it appeared that in most samples the pH changes would cease before net O_2 production ceased.

Table 1. Effect of CO_2 on light-induced pH changes in Euglena.

Sample No.	CO_2 conc. (%)	Dark pH change	Δ pH(-)	β	ΔH^+ total (μequiv $H^+/10^6$ cells)
1	0.00	7.01 - 6.35	0.66	0.074	0.05
	0.03	6.75 - 6.33	0.42	0.164	0.07
2	0.00	6.65 - 6.20	0.45	0.096	0.04
	0.03	6.34 - 6.14	0.20	0.560	0.07
3	0.00	6.72 - 6.32	0.40	0.076	0.03
	0.03	6.99 - 6.50	0.49	0.40	0.20
	1.0	6.70 - 6.32	0.38	0.70	0.25
4	0.00	6.35 - 5.96	0.39	0.074	0.03
	1.0	6.63 - 6.24	0.39	0.215	0.08

Suspension of photosynthetic Euglena were aerated at 3 ml/min and exposed to light-dark cycles. The CO_2 concentration indicated is the concentration of CO_2 in the stream aerating the sample. The dark pH change shows a typical decrease in pH in the dark under the conditions described, and is used in these calculations instead of the light pH change because the titration capacity was measured in the dark. Because of the repeating nature of the light-dark cycles the pH changes in the light were equal to the dark changes shown. ΔpH is the magnitude of the pH decrease. β is the titration capacity of each suspension in μequiv OH^- used/pH unit per 10^6 cells at the lower pH of the dark pH change. Cell concentrations/ml: 1 - 4.8×10^5; 2 - 4.5×10^5; 3 - 4.4×10^5; 4 - 5.3×10^5.

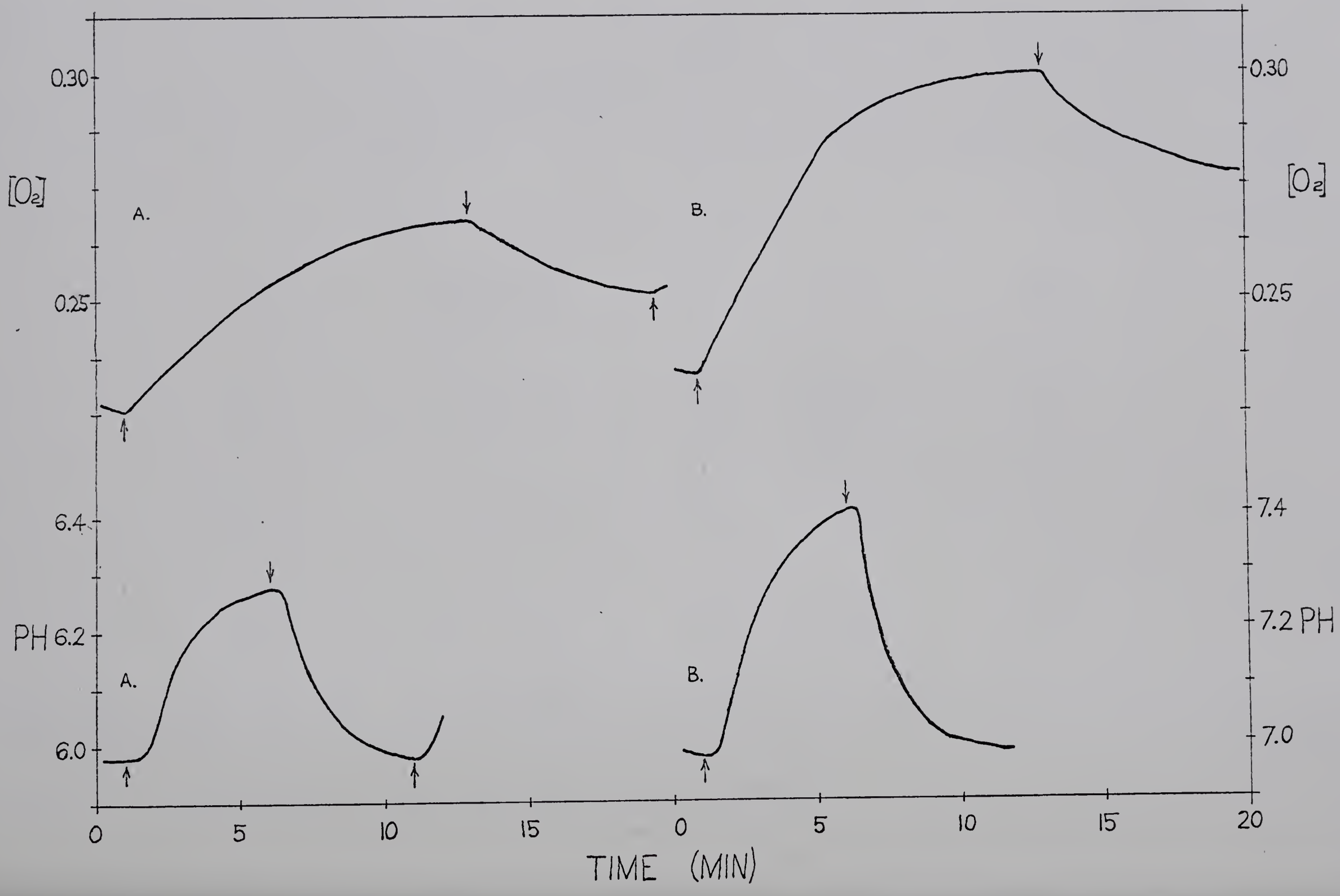

0.30
[O2]
A.
0.25
B.
0.30
[O2]
0.25
6.4
7.4
PH 6.2
A.
B.
7.2 PH
6.0
7.0
0
5
10
15
0
5
10
15
20
TIME (MIN)

Figure 9. Comparison of light-induced pH changes with oxygen uptake and evolution by Euglena. Consecutive pH and oxygen measurements were made on two suspensions of Euglena. The upper curves are tracings from the polarographic recordings while the lower curves show the pH recordings. (A) 4.2ml suspension (4.6×10^5 cells/ml) was used for pH measurements while aerated with CO_2-free air at 3ml/min. Total proton release in dark (5 min) = 0.03μequiv $H^+/10^6$ cells. 2.0ml of the same suspension was used for oxygen measurements. Photosynthetic O_2 evolution in light in first 5min (not corrected for respiration) = 0.07μmoles O_2/million cells. (B) 2.4ml suspension (4.4×10^5 cells/ml) was used for both measurements, and was aerated at 25ml/min with 0.03% CO_2 in air during pH measurements. Total proton release in dark = 0.07μequiv $H^+/10^6$ cells. Uncorrected photosynthetic oxygen evolution in the light in the first 5min = 0.135μmoles $O_2/10^5$ cells. [O_2] ordinate is in μmoles O_2/ml.

The time course of the light-dependent pH changes did not change with the addition of CO_2. The time course of the O_2 uptake does not show the lag seen in the pH changes, and often has a different length of the initial rapid rise period to that of the pH rise. A comparison of the time courses thus suggests that the two phenomena are not directly related.

Low aeration (5 ml/min) decreased the titration value of a suspension of cells to about two thirds that of an unaerated suspension, probably by lowering the amount of CO_2 and bicarbonate present. High aeration (25 ml/min) did not further change the titration value, although it did decrease the actual pH change in the light. Continuation of the high aeration for about 3 h completely inhibited the light-induced pH changes, showing that some CO_2 is necessary for their occurence.

3. Effects of Ethylene on Euglena

(a) Effects on Light-Induced pH Changes

Ethylene (100 ppm in CO_2-free air) was bubbled through Euglena suspensions that were exposed to 5 min light and dark cycles. The pH changes between consecutive cycles in the light and in the dark are shown in Table 2. When the ethylene treatment was started at the beginning of one dark cycle, the pH change between the next light cycle, and the preceding one, and that between the next two dark cycles increased significantly. Figure 10 shows a typical curve illustrating this effect, and also acts as a key for Table 2. The magnitude of the pH change was only slightly affected by this treatment, but there was a definite change in the steady-state pH values in both the light and in the dark. In the majority of cases, the pH increased with the treatment, although pH decreases were sometimes seen. The data in Table 2 are calculated without

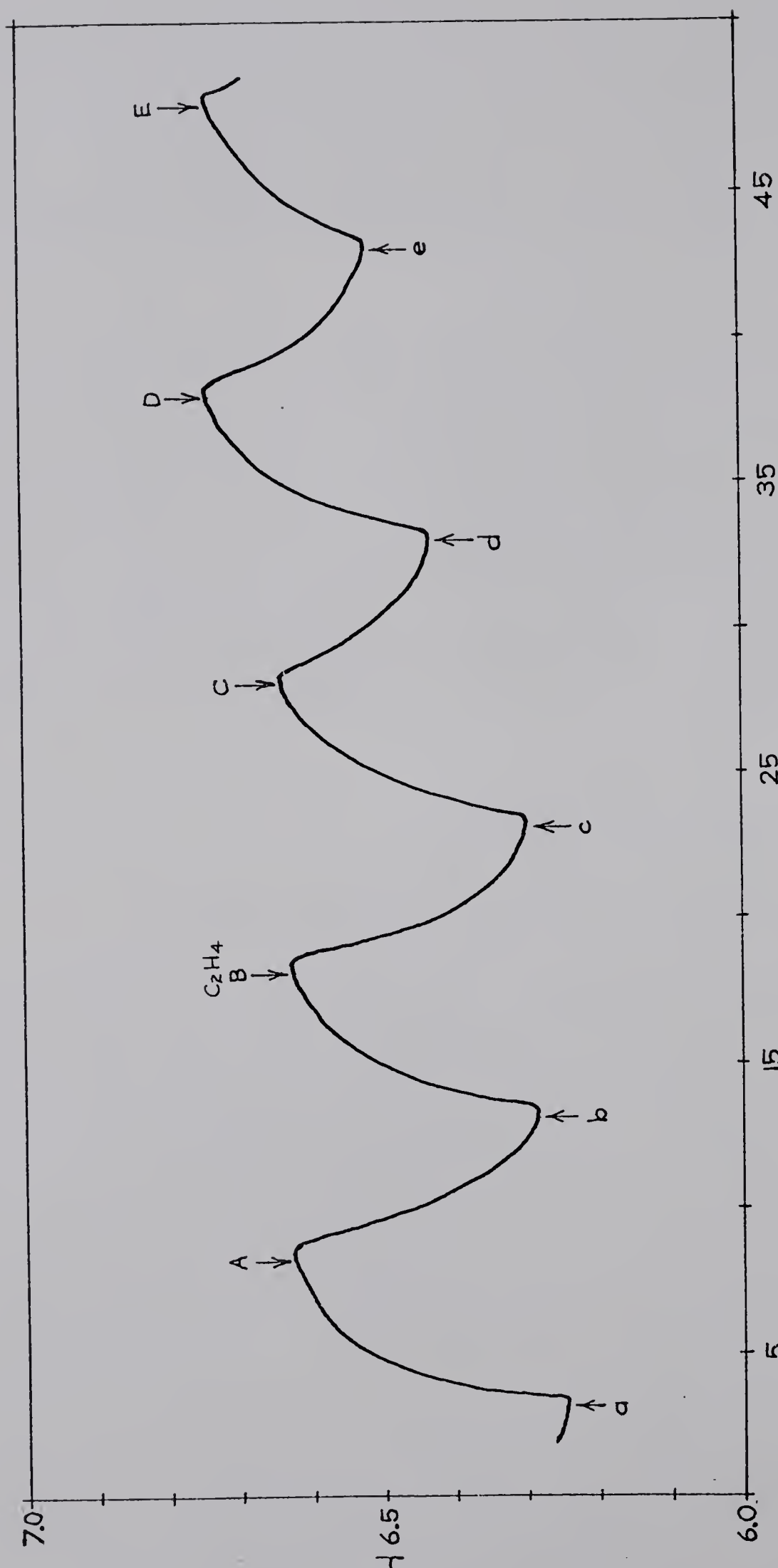


Figure 10. Effect of ethylene on light-induced pH changes in Euglena. A 3 ml suspension of Euglena (2.6 x 105 cells/ml) was aerated with CO_2 - and ethylene - free air (3 ml/min). At point B, the airstream was changed to 100 ppm ethylene. (↑) - light on. (↓) - light off.

Table 2. pH changes in Euglena suspensions on the addition of ethylene.

	Light values				Dark values			
* pH changes between	A - B	B - C	C - D	D - E	a - b	b - c	c - d	d - e
Mean difference in pH	0. 014	0. 025	0. 041	0. 023	0. 015	0. 030	0. 033	0. 022
± SE	0. 002	0. 005	0. 007	0. 004	0. 002	0. 005	0. 004	0. 005
Student's *t*		2. 0	3. 5	2. 0		2. 8	3. 7	1. 4
Degrees of freedom		50	50	40		58	58	45
P (%)		5	1	5		1	0. 01	10

* Letters refer to steady-state pH values as shown in Figure 10.

29 suspensions of Euglena were treated the same way as the example shown in Figure 10, with ethylene addition starting at B, the end of the second light cycle. The change in the steady-state pH between consecutive cycles in the light (upper case lettering) and in the dark (lower case lettering) was calculated. The means of these changes for the intervals before and at various times after the ethylene addition were compared statistically. The *t* and *P* values given are for the comparison of the indicated interval with the before treatment intervals (A - B or a - b) in its respective light or dark set.

regard for the sign of the change, and are from samples in the pH region 6.0 to 7.0. This variability may account for the comparatively high standard error of the pH changes. There was no pH change on changing the aeration of a sample of distilled water from CO_2-free air to the ethylene-air mixture.

Similar pH changes, possibly slightly greater in magnitude, were seen on addition of the ethylene-air mixture to suspensions of cells in constant dark. The pH change was complete in about 15 min, as was suggested by the results with the light-dark cycles.

To estimate how quickly the cells were actually exposed to ethylene by bubbling gas mixtures through the suspensions at 5 ml/min, the rate of solution of the gas in water was measured. More than 1 ppm ethylene was found after the gas mixture had bubbled through water for 10 sec. The level increased to 7.5 ppm after 4 min treatment. Equilibrium concentration of the gas, if the air above the solution were the same as the bubbling mixture, would be about 11.5 ppm (Olson and Spencer, 1968). Thus the cells were exposed to the gas relatively rapidly, and the delay in effect was not directly related to the slowness of solution of the ethylene.

Cells grown and tested at 28° gave larger and more frequently positive pH changes than those treated at 21°.

The effect of ethylene on the pH was largely reversible by air in both the samples treated during light and dark cycles, and in those treated in constant dark. Of a total of 18 samples tested, 10 showed ethylene-induced pH increases that were reversed by air, and 3 gave air-reversed pH decreases. Two showed pH increases that were increased by aeration, while the remaining 3 showed decreases with both

ethylene and aeration.

There was no reproducible change in titration values of the cells with ethylene treatment.

One thousand ppm ethylene in air did not give an appreciably greater response than did the 100 ppm routinely used.

(b) Effects on the Growth of the Cells

Two cultures of E. gracilis Z were grown at 21° under LD;14, 10 and aerated at 50 ml/min with 10 ppm ethylene in air and 120 ppm respectively. There was no significant difference in growth rate or cell size between the treated cultures and untreated controls during 4 days growth. The cell concentrations increased from 8×10^3 to 5×10^4 cells/ml for the 10 ppm culture and its control and from 10^4 to 10^5 cells/ml for the 100 ppm culture and its control.

(c) Production of Ethylene by Euglena

The ethylene production by the cells was measured to show if the gas would be normally present in their environment. The basic 500 ml light-synchronized culture grown at 21° and aerated with 1% CO_2 in air at 50 ml/min was used. In 4 measurements, levels of about 0.01 nl/10^6 cells per h were obtained above a constant background level equivalent to 0.02 nl/10^6 cells per h. This compares with reported levels equivalent to 2×10^{-11} nl/10^6 cells per h reported for valencia oranges, and 7×10^3 for passion fruit (Burg, 1962). (The weight of one Euglena was taken to be 3×10^{-9} g. (Edmunds, 1965b).)

The observed rate of ethylene production would result in a build-up of 0.01 ppm ethylene per h in a culture of a concentration of 10^6 cells per ml. This contrasts with the concentration of 11.5 ppm ethylene in solution used to obtain pH changes in Euglena suspensions.

4. The Effects of Addition of Salts on Light-Induced pH Changes in Euglena

The effect of changing the ionic environment of the cells on the light-dependent pH changes was tested. NaCl, KCl, $MgCl_2$, and $CaCl_2$ gave decreases of from 0.05 to 0.20 pH units when added to a final concentration of 10 mM. Like the ethylene effects, they did not change the time course of the light-dependent pH changes. The effect was the same whether the salts were added as 1.5 M or 0.07 M solutions.

Addition of salts to distilled water often gave a small response similar to that seen with cells present, likely because of the slight acidity of the salt solutions (see p-20). This pH change was completely absent when a phosphate buffer of a similar titration capacity to a Euglena suspension was tested in place of the cells.

The effects of salts were tested both in cells in constant dark, and in suspensions exposed to alternating light-dark cycles. Typical recorder tracings of the responses in dark are shown in Figure 11. The response to $CaCl_2$ (and also to $MgCl_2$) is slower to reach a final pH than in the response to KCl. Similar results were found in the two systems, although there was a somewhat greater sensitivity of cells in constant dark. The response in both cases did not occur after a threshold concentration, about 20 mM for KCl and NaCl, and 10 mM for $CaCl_2$ and $MgCl_2$, had been reached.

KCl and NaCl did not affect the pH of cells in light-dark cycles, but gave pH changes in suspensions in constant dark, as is shown in Table 3. Ethylene eliminated much of the salt-induced change (compare also Figure 11 A and C). Details of the ethylene treatments are shown in Table 4. The data in Table 5 give some information about the time course of this inhibition of pH change by high levels of ethylene. They suggest that the gas actually reverses the pH change

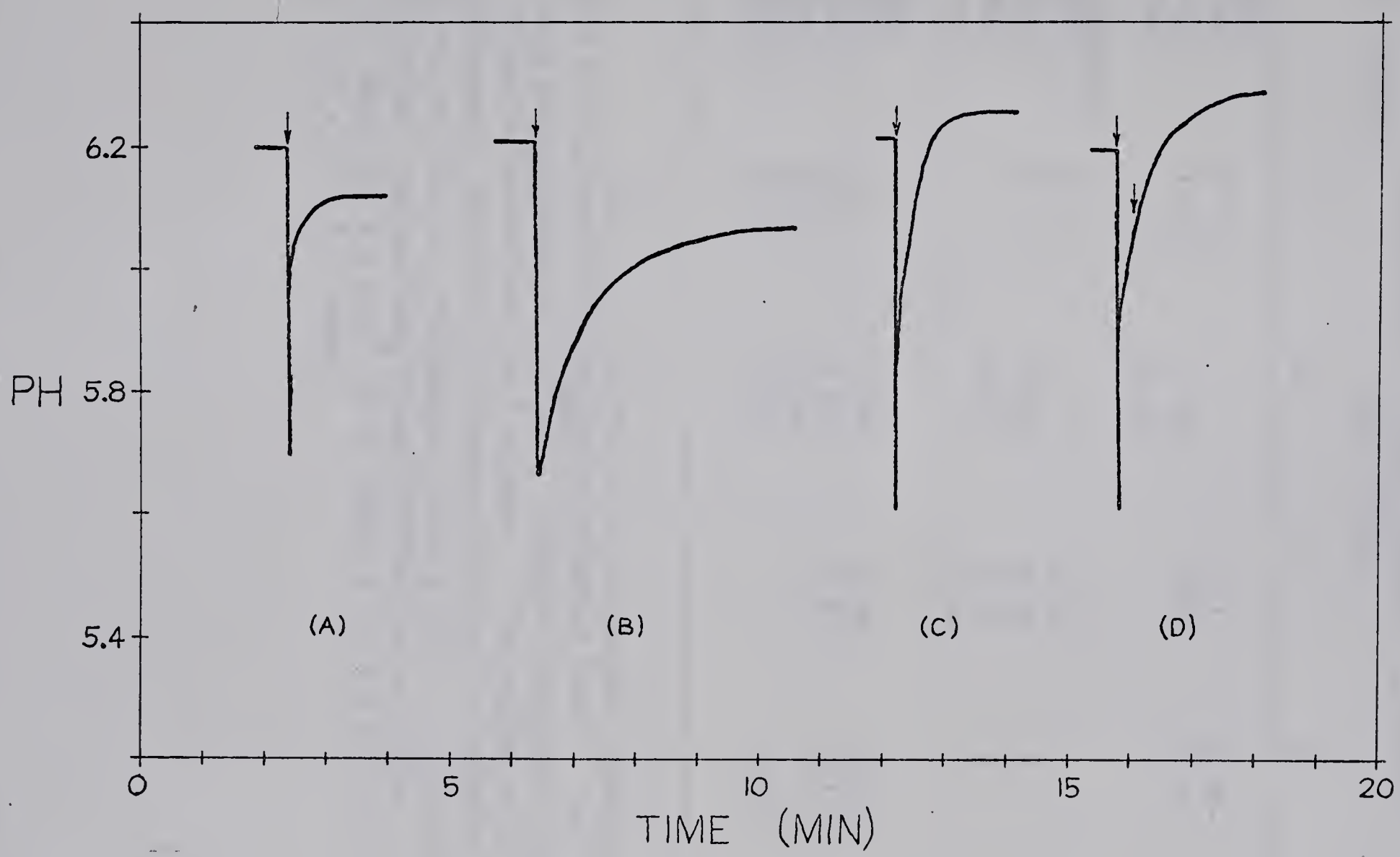


Figure 11. pH changes in Euglena suspensions on addition of salts. KCl (curves A, C, and D) and $CaCl_2$ (curve B) to give a 2 mM concentration were added to suspensions of photosynthetic Euglena (1 to 5 x 10^5 cells/ml) at the time indicated by the arrow. For C, the suspension was pretreated by bubbling with air containing 100 ppm ethylene for 15 min (final concentration -10 nl/ml). For D, ethylene to a final concentration of 4300 nl/ml was added at the second arrow; 10 sec after KCl addition.

Table 3. Effects of NaCl, KCl and ethylene on the pH of Euglena suspensions.

Treatment	Change in H^+ conc. (μequiv $H^+/1 \times 10^4$) Run Number			
	1	2	3	4
air				
NaCl	0		1.71	0.05
KCl	1.15	1.25	0.81	0.02
NaCl		0.30		
ethylene				
KCl	0		0.50	
NaCl	0	0.53	0.17	0
KCl		0.41	0.36	0
NaCl			-0.18	
air				
KCl	1.3	0.55	0.94	0.03
NaCl	0.3	0.63	0.42	-0.01
KCl	0.21	0.79		
NaCl	0.43	-0.38		+0.01

Each run consisted of the sequential addition of NaCl and KCl to a suspension of Euglena in distilled water. Each addition gave 2 mM concentration of the salt. Ethylene treatment was by bubbling 100 ppm ethylene in air at 3 ml/min. The lengths of the ethylene treatments, and of the air treatments before the addition of salts are shown in Table 4. All samples were at 21°. Cell concentration (cells/ml), approximate pH, and lighting for each run: 1 - 8.8×10^5, 6.0, dark; 2 - 4.0×10^5, 6.3, light; 3 - 3.4×10^5, 6.1, dark; 4 - 4.0×10^5, 7.5, dark.

Table 4. Ethylene and air treatments for samples shown in Table 3.

Treatment	run no.	length of treatment (min)	pH		change in H^+ conc. (μequiv $H^+/l \times 10^4$)
			before	after	
ethylene	1	17	5.95	6.00	-1.40
	2	12	6.44	6.41	0.16
	3	9	6.07	6.16	-1.60
	4	19	7.40	7.54	-0.11
air	1	17	6.00	5.97	0.50
	2	13	6.33	6.34	-0.10
	3	10	6.12	6.11	0.18
	4	12	7.59	7.51	0.05

Details of KCl-and NaCl-induced pH changes in each run are shown in Table 3. Ethylene treatment was by bubbling 125 ppm ethylene through the suspension at 3.0 ml/min. Air treatment consisted of bubbling ethylene-free air at the same rate.

Table 5. Effect of KCl and ethylene on the pH of Euglena suspensions.

gas treatment	time of salt addition (sec)	pH before	pH after	change in H^+ conc (μequiv H^+/1 x 10^4)	
air		5.88	5.95	-1.9	
		5.85	5.75	3.6	
		5.85	5.76	3.2	
		5.98	5.92	1.6	(a)
		6.33	6.36	-0.3	
		5.86	5.70	6.1	
		5.98	5.90	2.1	
		6.19	6.18	0.15	
		5.84	5.84	0.0	
ethylene	30 after gas	5.75	5.77	-0.8	
		6.13	6.15	-0.34	(a)
ethylene	with gas	5.97	6.06	-2.0	
		6.18	6.25	-1.0	
ethylene	10 before gas	6.01	6.06	-1.07	
		5.78	5.78	0.0	
ethylene	15 before gas	6.02	5.93	2.2	
		6.24	6.31	-0.86	

All ethylene treatments consisted of the addition of 300 µl ethylene-saturated water (final concentration-4300 : nl ethylene/ml suspension). All samples were treated with 10 µl 1.5 M KCl (final concentration-2 mM) and the resulting pH change was measured. Each set of figures represents a different sample of cells (about 2 x 10^5 cells/ml) in distilled water. All cells were from the same synchronized sample. All samples were in the dark except for the two marked (a).

caused by KCl, and that it must be added within about 15 sec after the KCl in order to be effective.

$MgCl_2$ and $CaCl_2$ caused pH changes both with cells exposed to light-dark cycles, and with those in constant dark. Examples of the former effect are shown in Figure 12. The change in pH was about 40% greater in the light than in the dark in 8 out of 9 experimental runs. Ethylene (100 ppm in air) treatment for 15 min before addition of the salts increased the pH change caused by $MgCl_2$ in cells undergoing light-dark cycles, but did not affect pH changes caused by $CaCl_2$, or those by either salt in cells in constant dark.

$MgSO_4$ and K_2SO_4 also gave pH changes both in cells in the constant dark, and in those in light-dark cycles. The effect is thus not dependent on the presence of the Cl^- ion. Both salts were ineffective at concentrations greater than 10 mM.

The effect of salts on the titration capacity of the suspensions was not measured because of difficulties in obtaining reliable data with the slow response of the system.

Interactions of Euglena with added salts and ethylene may also be followed by measuring the changes in absorbance of stirred suspensions. Exploratory experiments suggested that this method might be valuable in future studies of the system.

Addition of KCl to a final concentration of 3.75 mM gave a decrease in absorbance that was stable with time in 3 out of 4 measurements (0.01 OD in a suspension of total OD of 0.245 (about 2×10^5 cells/ml)). There was an initial overshoot in the decrease of absorbance of 0.003 OD, and the final value was reached in approximately 90 sec.

Addition of ethylene (final concentration of 3000 nl/ml)

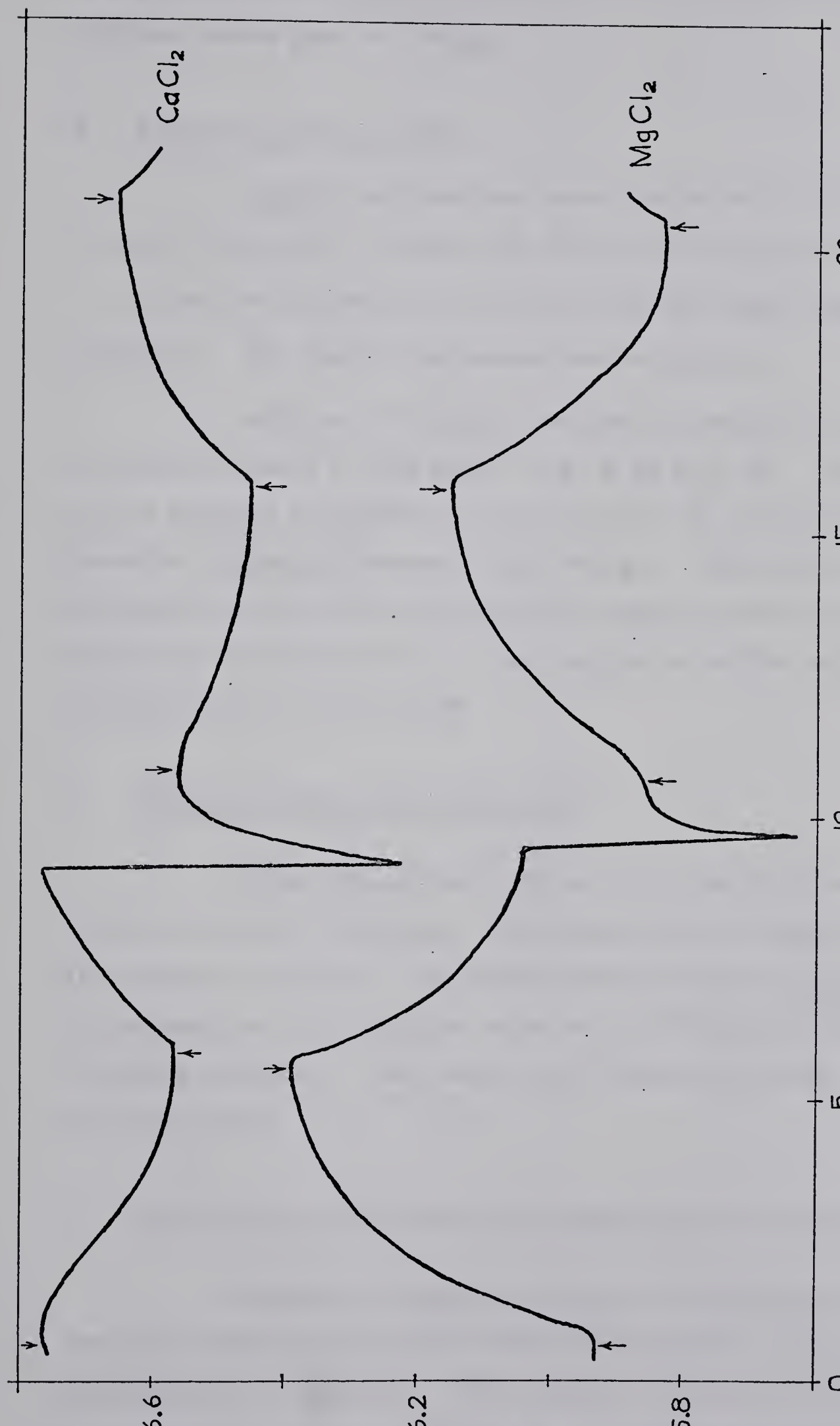


Figure 12. Addition of $CaCl_2$ and $MgCl_2$ to *Euglena* suspensions exposed to light-dark cycles. The abrupt discontinuity in the curves occurred on addition of $CaCl_2$ (top) or $MgCl_2$ (bottom) to different suspensions of photosynthetic cells (5.4×10^5 cells/ml). Final salt concentration was 6 mM. Upright arrows: light on; inverted arrows: light off.

simultaneously with the KCl did not inhibit the decrease, but resulted in a return to the original OD within 2.5 min (2 out of 4 samples). Ethylene alone gave no change.

5. Effects of Triton X-100

Euglena suspensions were treated with Triton X-100, a non-ionic detergent, to test the effect of the presumed alteration of the surface properties of the cells on the light-induced pH movements. The results are summarized in Table 6.

Addition of 50 µg/ml suspension caused a sharp pH decrease of about 0.3 pH units, e.g. 6.24 to 5.94. One hundred µg per ml gave a ten-fold decrease in total H^+ movements, largely because of a large decrease in pH change. This concentration of detergent inhibited the photosynthetic oxygen uptake of a similar sample of cells about 15%. The respiration of the cells was not affected by the Triton X-100.

6. Titration Value of the Total Cell

If the chloroplasts act as a H^+ sink, they would draw protons from the cytoplasm, which would in turn draw protons from the external medium. The total titration value of *Euglena* was determined by dissolving the cells in 1.0 N NaOH, and titrating the resulting solution. This value was 3 times that of the intact cells in distilled water.

C. Light-Induced pH Changes in *Euglena gracilis* var *bacillaris* SM-L1

Samples of *Euglena gracilis* var *bacillaris* SM-L1, a streptomycin-bleached strain that lacks chloroplasts, were tested for light-dependent pH responses. The response of the first culture tested (4 samples) is shown in Figure 13. The characteristics of these pH

Table 6. Effects of Triton X-100 on light-induced pH changes and oxygen evolution by Euglena

Sample No.	Triton X-100 (µg/ml)	pH change in dark	β	ΔH^+ total (µequiv $H^+/10^6$ cells)	O_2 * (+)L	(-)D
1	0	6.27-5.98	0.08	0.03		
	100	5.57-5.53	0.05	0.003		
2	0	6.75-6.37	0.07	0.03		
	200	5.56-5.53	0.05	0.003		
3	0				0.10	0.016
	100				0.085	0.016
	200				0.085	0.016

* µmoles $O_2/10^6$ cells for 5 min after light turned on. (+)L - oxygen evolution (-) D - oxygen uptake

Triton X-100 was added to suspensions of Euglena in distilled water that were undergoing light-dark cycles. The pH change in the dark is the change in pH during 5 min after the light was turned off. β is the titration capacity in µequiv-OH^- used/pH as determined from pH change on addition of a known amount of base to cells in the dark. ΔH^+ total shows the total H^+ movements during the dark cycle noted. Cell no./ml : 1 - 5.1×10^5; 2 - 5.3×10^5; 3 - 3.8×10^5.

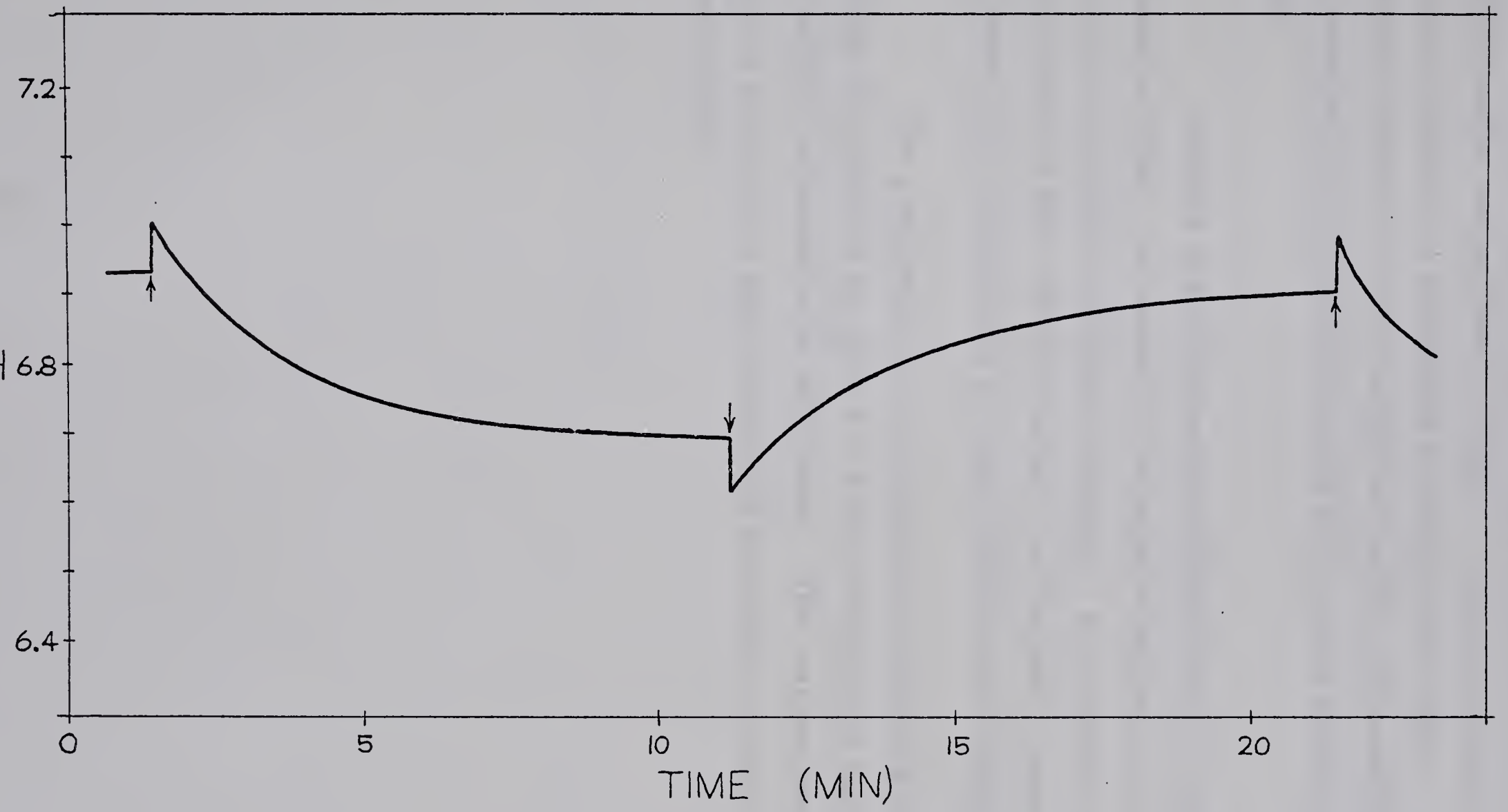


Figure 13. pH changes in a suspension of Euglena gracilis var bacillaris SM-L1 in light-dark cycles. A suspension of cells in distilled water (2.3 ml x 4.8 x 10^5 cells/ml) was exposed to 10 min light-dark cycles. The suspension was stirred, at 28°, and aerated with CO_2-free air at 25 ml/min. There were no light-induced pH changes during the first 30 min after the start of measurements.

changes, which are opposite in direction to those seen with photosynthetic cells, are outlined in Table 7. Figure 14 shows the titration capacity of a suspension of the pH-active cells. The small difference between the cells in light and dark might result from the high aeration (25 ml/min) used during the titration and pH measurements.

Samples of cells from later cultures did not show a pH response to light. In contrast to the cells in the first culture, these cells were non-motile and lacked eyespots. It is possible that the eyespot was lost after the first culture, as frequently happens when colourless cells are grown in the dark (Huntner and Provasoli, 1955).

The respiration of the streptomycin-bleached cells was inhibited 40 to 60% for the first 2 to 3 min the light was turned on, after which it returned to the dark level. This was observed with cells that did not show light-induced pH decreases, as well as with active samples.

Table 7. Comparison of light-induced pH changes by E. gracilis, Z and streptomycin-bleached E. gracilis var bacillaris SM-L1.

	E. gracilis Z	E. gracilis var bacillaris SM-L1
Extent of light-induced change (μequiv $H^+/10^6$ cells)	0.1	0.16
Direction of light-induced change	pH increase	pH decrease
Titration capacity (μequiv OH^- used/pH per 10^6 cells) pH 6.0 to 8.0	0.10-0.32	0.10-0.28
Initial lag time (sec)	20-30	sudden pH increase (light) sudden pH decrease (dark)
Time to 75% total pH change in light (min)	2.0	3.5
Time to 75% total pH change in dark (min)	3.0	4.1
Time from beginning of light-dark cycles to first pH change (min)	0.3	about 30

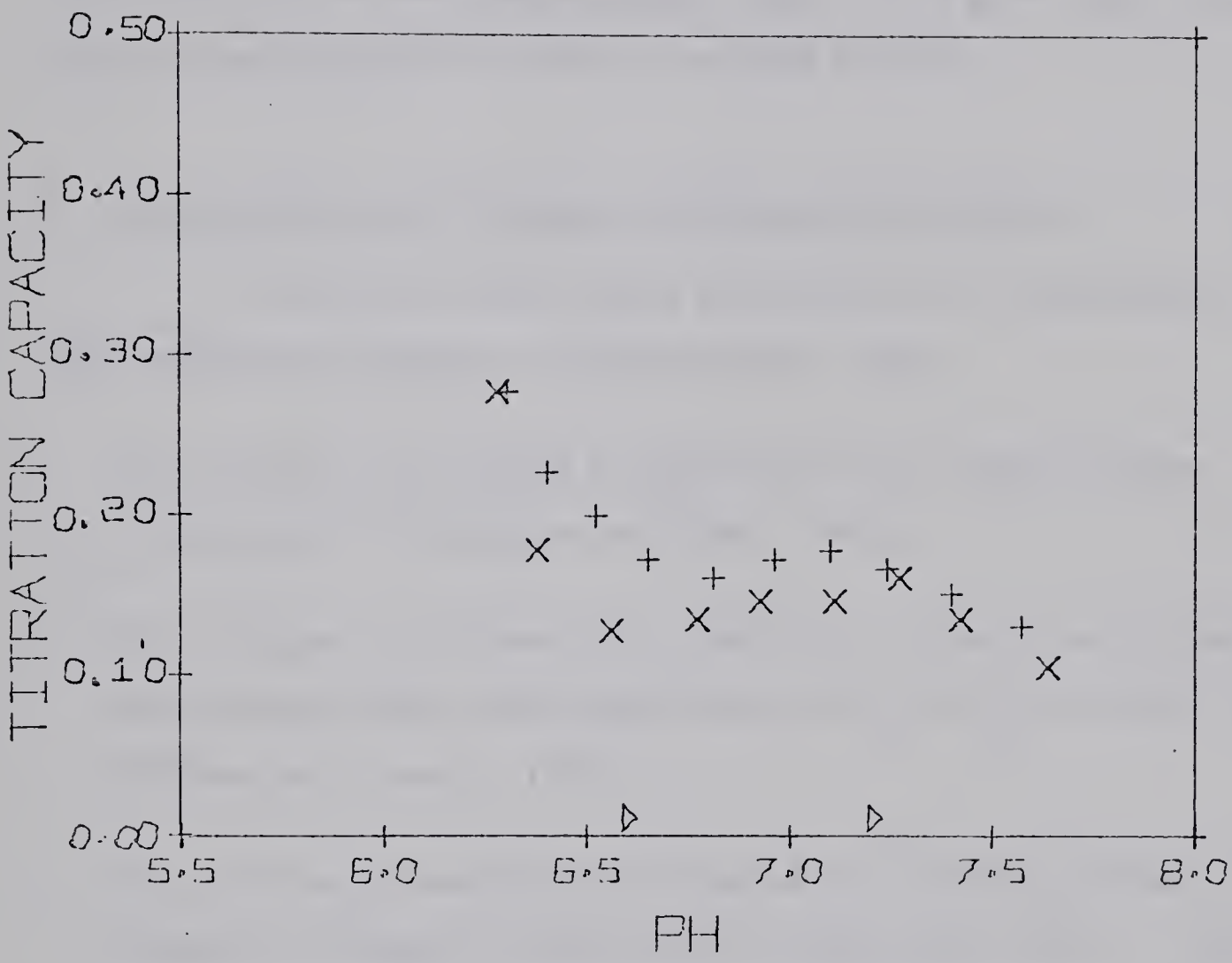


Figure 14. Titration capacities of a suspension of E. gracilis var bacillaris SM-L1 in distilled water.

Titration capacities are in μequiv OH^- used/pH per 10^6 cells. A 3 ml suspension (2.5×10^5 cells/ml), aerated at 25 ml/min, was titrated with 0.003 N NaOH. The cells were obtained from a batch culture (1.8×10^4 cells/ml) grown in 0.05 M Na acetate medium at 28° with 25 to 50 ml/min aeration and low light.

\+ Light for 10 min before titration

x Dark for 18 min before titration

DISCUSSION

Four types of pH changes in suspensions of Euglena in distilled water have been considered in this thesis - those caused by light, by ethylene and by salts in photosynthetic cells, and those caused by light in non-photosynthetic cells. All give some insight into the characteristics of the cell and its surface.

A. Light-Induced pH Changes in Photosynthetic Cells

There are three major possibilities for the origin of light-induced pH changes in photosynthetic cells.

1. The changes are related to light-induced H^+ uptake by the chloroplasts (Schuldiner and Ohad, 1969).

2. The changes result from CO_2 uptake and release by the cells by causing shifts in the equilibrium: $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$ (Atkins and Graham, 1971).

3. The pH change results from changes in H^+ binding by the cells related to changes in the metabolic state of the cells. This is similar to the mechanism supported by Murakami and Packer (1970) for light-induced pH changes by chloroplast fragments.

The data in this thesis suggest that the light-induced pH changes with Euglena gracilis Z can be explained by a combination of the last two alternatives.

1. Relation to H^+ Uptake by Isolated Chloroplasts

The idea that light-dependent pH changes in intact cells are directly related to light-dependent pH changes in isolated chloro-

plasts (Schuldiner and Ohad, 1969) is not supported by the data in this

Firstly, a much larger decrease was seen in light-induced H^+ movements than in photosynthetic oxygen evolution on the addition of Triton X - 100. This indicates that photosynthetic electron transport that is closely linked to photosynthetic oxygen production is not directly connected with light-induced pH changes.

Secondly, changes occurring in the chloroplast would not be expected to give the observed changes in titration capacity of the intact cells.

Thirdly, protons released by the chloroplasts would likely be buffered by the cytoplasm, and only a small effect would appear outside the cell. The total H^+ movements observed in the absence of CO_2 were similar in magnitude to those seen in isolated chloroplasts (Jagendorf and Uribe, 1966), and show no indication of intracellular buffering.

Fourthly, current views of the role of a proton gradient in energy production state that a potential gradient, and not a proton gradient, is present during on-going phosphorylation (Gre ville, 1969). The chloroplasts may act as a H^+ sink only very briefly at the beginning of photosynthesis, and would not cause a permanent change in pH external to the cells.

2. Relationship to the Photosynthetic Carbon Dioxide Metabolism

Atkins and Graham (1971) showed that for Chlamydomonas reinhardi in the closed system of a Clark electrode and in the light, the ratio of CO_2 added : O_2 evolved : H^+ used was 1:1:1. They suggested that the pH changes resulted from changes in the equilibrium:

$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$, as CO_2 was taken up by the cells. This concept is not applicable to the open system used in these studies of Euglena where the amount of CO_2 dissolved is also determined by the equation: CO_2 (dissolved) = 3.43×10^{-2} pCO_2, where pCO_2 is the partial pressure of CO_2 in atmospheres (Butler, 1962), and thus CO_2 taken up by the cells can be replaced by CO_2 from the air stream.

One feature of Atkins and Graham's data suggests that their hypothesis might be an oversimplification of the situation in closed systems. Their experimental situation was simply to add known amounts of CO_2 dissolved in water to a suspension of cells that had reached a steady pH in the light. This caused a simultaneous H^+ uptake and O_2 evolution which both stopped at the CO_2 compensation point. However, the pH increase by the cells was only about two-thirds the decrease in pH resulting from the addition of CO_2. Either the assumption that the CO_2 added was completely taken up by the cells is incorrect, or some additional movements of H^+ had occurred.

It should be noted that the CO_2 added: O_2 evolved: H^+ uptake ratio during the induction phase of photosynthesis (about the first 5 min) was not measured by Atkins and Graham (1971) who were adding CO_2 to cells all ready adjusted to the light. During the induction phase of photosynthesis, CO_2 uptake does not necessarily equal O_2 production (Rabinowitch, 1956). Thus only very careful measurements during this phase could show the relationship between CO_2 uptake: O_2 produced: H^+ uptake.

As well as appearing unlikely because of equilibria in an open system, the direct involvement of CO_2 in light-induced pH changes does not explain the existence of a steady pH in the dark, nor the time course of the dark-induced pH decrease. The initial photosynthetic rate of oxygen evolution was several times larger than the respiratory oxygen uptake, but the dark-induced pH decrease was only about 1.5

times slower than the increase in the light.

3. Relationship to Metabolic Changes During the Commencement of Photosynthesis in the Cell

The large variation in H^+ movements seen at different concentrations of CO_2 might represent, at least in part, differences in the intensity of the changes during the induction phase of photosynthesis. Rabinowitch (1956) considered the first 2 to 5 min of photosynthesis in both higher plants and algae to represent an induction phase, characterized by changing rates of oxygen evolution and CO_2 uptake. He suggested that this period might represent the readjustment of stores of respiratory and photosynthetic intermediates, and deactivation and reactivation of enzymes to prepare the cell for active photosynthesis. Changes in the conformation of membrane macromolecules to cause permeability changes might also occur (Cerbón 1970). It is suggested that the light-induced pH changes in Euglena result from changes in proton binding by the cells during the above transitions. pH changes resulting from actual CO_2 movements may also occur, although their relative importance cannot be assessed from the data in this thesis.

This hypothesis would explain the observed light-dark changes in titration capacity of the cells since different numbers of buffering groups might be exposed at the cell surface in each state. It also fits the observed steady pH in constant light or dark. Since the dark-induced pH decrease would be related to intracellular changes during the photosynthesis-respiration transition, the time course would not have to be related to the respiratory rate. The large increase in the dark-induced pH decrease in the presence of CO_2 even though the respiration rate is unaffected is also supported by this hypothesis.

The pH changes by bleached E. gracilis var bacillaris

SM-L1 can also be explained as changes in proton binding by the cells during a change in the metabolic state of the cells. Because this mechanism is just a generalized shift in H^+ binding, protons can either be released or taken up by the cells in the light according to the nature of the metabolic change and the condition of the cells before the change. The actual nature of this light-induced metabolic change in colourless cells is not known.

The occurrence of the light-induced pH changes in the colourless cells was dependent on some specific condition of the cells, possibly the presence of the eyespot. The role of the eyespot in Euglena is unknown, since cells that lack this organelle are still phototropic (Huntner and Provasoli, 1955). The actual light receptor in the cell may be a swelling at the base of the flagellum, which is visibly colourless. It may not be necessary for a photoreceptor to be brightly coloured, especially one which might respond to infrared radiation.

One definite suggestion that has been made concerning intracellular changes at the beginning of photosynthesis is the movement of organic acid anions into the chloroplasts, which results in light-induced shrinking of these organelles (Packer, Deamer and Crofts, 1966, p-281). Nobel et al (1969) showed that pea chloroplasts in vivo required almost 10 min to reach maximum flattening at 310 ft-c. The pH changes external to intact cells could be caused in part by conformational changes in cell membranes resulting from the movement of these substances away from the membranes.

B. Effects of Ethylene on the pH of Euglena Suspensions

Ethylene produced small pH changes in suspensions of Euglena. This effect may result from the gas localizing in the hydro-

phobic areas of the cell membrane, and causing a change in the strength of proton binding by the membrane macromolecules.

Kilkson (1969) suggested that the relative strength of lipid-lipid and lipid-protein interactions of a membrane could be varied by the introduction of lipid-soluble molecules. He postulated that this would change the lipid surface energy, and thus the conformation of the membrane. This is an example of a possible means of action of ethylene.

The relative slowness of the action of the gas suggests that either some permeability barrier is present, or that a certain level of the gas must accumulate in one area of the cell before any effect is seen. This possible need for an accumulation of ethylene to cause a pH change, and the very gradual nature of the change supports the previous suggestion that ethylene is merely collecting in the membrane lipids. The binding of a compound at a definite site will often be magnified in effect by co-operative transitions of the neighbouring molecules (Changeux, 1969; Ling, 1962).

The variability in the response to ethylene from pH increases to pH decreases shows that the action of the gas was dependent on the condition of the cells and their membranes. Small variations in the washing of the cells, which would affect their ionic environment, and in the growth conditions, particularly in the concentration of CO_2 used, which might change the composition of the membrane slightly, might alter the response of the cells to the gas.

The level of ethylene active in producing pH changes in the cells is of the same magnitude normally associated with the hormonal activity of the gas. It is possible that the activity is dependent

only on the lipid solubility of the gas, and not on any specific molecular characteristics, and that other similar gases such as ethane would have the same effect as ethylene. Ethylene is important because it is produced by the cell, and conceivably could be concentrated within the cell. Although these studies did not show any gross physiological effects of the gas, it is possible that changes caused by the gas might be important in the cells' ability to resist environmental stress.

C. Effects of Salts on the pH of Euglena Suspensions

Salts were able to cause pH decreases in Euglena suspensions, possibly by displacing H^+ bound to membrane macromolecules. The cells may lose membrane-bound salts during washing, and the effect seen may reflect a return to a more normal level.

The first addition of salts to a cell sample showed a large initial release of H^+, much of which was taken up again by the cells within 2 min. This overshoot was also seen in the changes in absorbance with the addition of salts. This suggests that the initial release of protons causes conformational changes in membrane macromolecules that result in reuptake of a portion of the H^+ to give a final stable state.

KCl and NaCl were the least active salts in causing a pH change, having an effect only on cells in the constant dark or constant light. KCl released from the reference electrode was present in the suspension at low levels, and might be expected to decrease the effectiveness of added salt. Na^+ is normally present in the cell only in trace amounts, and would not be expected to bind strongly to cell proteins (Ling, 1962). The absence of pH changes on addition of NaCl and KCl to cells undergoing light-dark cycles might be related to the changing ion fluxes occurring at the beginning of photosynthesis.

Mg^{++} and Ca^{++} are important intracellular regulators with

specific binding properties giving them biological activity, e. g. the role of Ca^{++} in muscle contraction. The sites of the membrane able to bind these salts might be available even during the light-dark cycles since their binding is probably stronger than that of Na^{+} and K^{+}.

The possibility of anions also being important in the salt-induced pH changes should not be omitted. Uptake of these ions could result in changes in the membrane H^{+} binding properties.

D. Effect of Both Salt and Ethylene on the pH of Euglena Suspensions

Ethylene inhibited the action of NaCl and KCl, the least active salts in causing pH changes in cell suspensions. The gas might change the macromolecular interactions of the membrane enough to resist the small stress from these salts, but not enough to change the action of $MgCl_2$ and $CaCl_2$. The reversal of the salt-induced pH change at high levels of ethylene may show that the salt altered the membrane enough to let ethylene be unusually effective. The necessity of adding the gas within 15 sec of the addition of the salt suggests that the gas is most effective during the temporary loss of H^{+} on addition of ions.

E. Conclusions

This thesis suggests that extracellular pH changes seen in Euglena suspensions in response to different stimuli may be indicators of changes in the cell membrane and possibly of changes in the cell metabolism. This is based on the following evidence:

1. Light causes a dark-reversible uptake of protons by the cells, and the pH of a suspension in light or dark is stable. This uptake is greatly increased by CO_2, possibly through a stimulatory effect on photosynthesis. Bleached cells also show light-induced pH changes.

2. Salts, especially $MgCl_2$ and $CaCl_2$, which have high biological

activity, caused a proton release by the cells. This likely resulted from the binding of the salts at the cell surface.

3. Ethylene at 10 ppm concentrations in the solution resulted in small pH changes in the suspensions, once again probably by changing the proton binding by the cells.

4. Ethylene was able to change the effect of NaCl and KCl on the suspension pH, showing that both the salts and the gas may at least in part have the same site of action, most likely the cell surface.

F. Further Work

Further work should be directed in two ways: to increase the experimental basis of the hypothesis advanced, and to utilize the techniques in further investigations of the properties of the cell surface.

The major problem that is unsolved is the relationship between the CO_2 uptake by the cells and the light-induced pH changes. Careful measurement of CO_2 uptake during the induction period of photosynthesis could in part clarify this issue. Measurements at different temperatures, and using various substances that might alter the cell surface without changing respiration and photosynthesis would also be valuable. (It is possible that salts may be in this category, and that they change the titration capacity as well as the pH of the suspension.) The use of metabolic inhibitors to alter the rate of metabolism would be limited by possible direct effects of these substances on the cell membrane. The possibility that Euglena may be capable of storing CO_2 intracellularily should be remembered in an investigation of the CO_2 metabolism of the cells.

The second problem to be considered regarding light-induced pH changes is the basis of the light-induced pH change observed in bleached cells, and the reason that only one culture of these cells was

active. If the hypothesis that the light was able to cause a metabolic change in the cells is correct, then careful measurements of CO_2 exchange by active cells might be able to show a light-induced change. Examination of the rates of uptake of the carbon source during dark-light transitions might also be useful.

Alteration of the composition of the cell and its membrane by starvation, and by heterotrophic nutrition might give useful information on the relationship of light-induced pH changes to the metabolic state of the cells.

The effect of ethylene on cells treated in the ways discussed above could be measured both by pH changes, and by changes in absorbance and light-scattering of the suspensions. This might define the properties of the membrane necessary for the activity of the gas. The effect of ethylene at different levels of CO_2 would also be of interest because of the inhibitory effect of the latter on the former in many systems. Gases with similar molecular and solubility characteristics should also be checked for their ability to cause pH changes.

The effect of salts on the pH and titration capacity of cells treated as discussed above might clarify the specific characteristics of the action of each salt. The effects of ethylene on the salt action would be a useful part of such investigations.

Further studies on the pH changes in Euglena suspensions should help clarify the interrelationships between the metabolic activity of the cell and the surface characteristics and permeability of the membrane. Possibly it might even show the effect the living state has on the cell macromolecules and their interactions.

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APPENDIX A

CALCULATION OF LIGHT-INDUCED H^+ MOVEMENTS

The amount of protons involved in a pH change in a buffered solution is the sum of the amount of change in H^+ concentration that would occur in an unbuffered solution plus the amount of H^+ actually used in the buffering action. The former is simply a calculation of the change in amount of ions between two known concentrations while the latter is the product of the titration capacity (μmoles OH^- or H^+ used/pH) and the pH interval over which the change occurred. (In this calculation it is assumed that the titration capacity measured by NaOH would be the same if measured by an acid, i. e. μequiv OH^- used = μequiv H^+ used). When, as in Euglena suspensions, the titration capacity varies with pH, the titration capacity value in the region of pH change under consideration is used to give an approximate answer.

The following is a sample calculation.

A. Data

1. A 3 ml sample (2.7×10^5 cells/ml) showed a pH decrease in the dark from 6.95 to 6.74.

2. 2 μl of 0.0032 N NaOH (0.0064 μmoles) gave a pH change in the dark from 6.74 to 6.80.

B. Change in Protons in an Unbuffered Solution

1. Change in H^+ conc $= (10^{-6.74} - 10^{-6.95}) \frac{\text{equiv } H^+}{\text{litre}} \times \frac{1}{0.27 \times 10^6 \text{ cells}}$

$= 0.256$ μequiv H^+/litre per 10^6 cells

2. For a 3 ml sample, this is a change of

$0.256 \times \frac{3}{1000} = 7.68 \times 10^{-4}$ μequiv H^+.

C. Amount of H^+ Used by the Buffer

1. Titration capacity = $\frac{0.0064 \text{ μequiv } OH^- \text{ used}}{(6.80-6.74) \text{ pH}} = 0.107$

2. Amount H^+ used for observed pH change from 6.95 to 6.74 (0.21 pH)

$= 0.107 \frac{\text{μequiv } OH^- \text{ used}}{\text{pH}} \times 0.21 \text{ pH}$

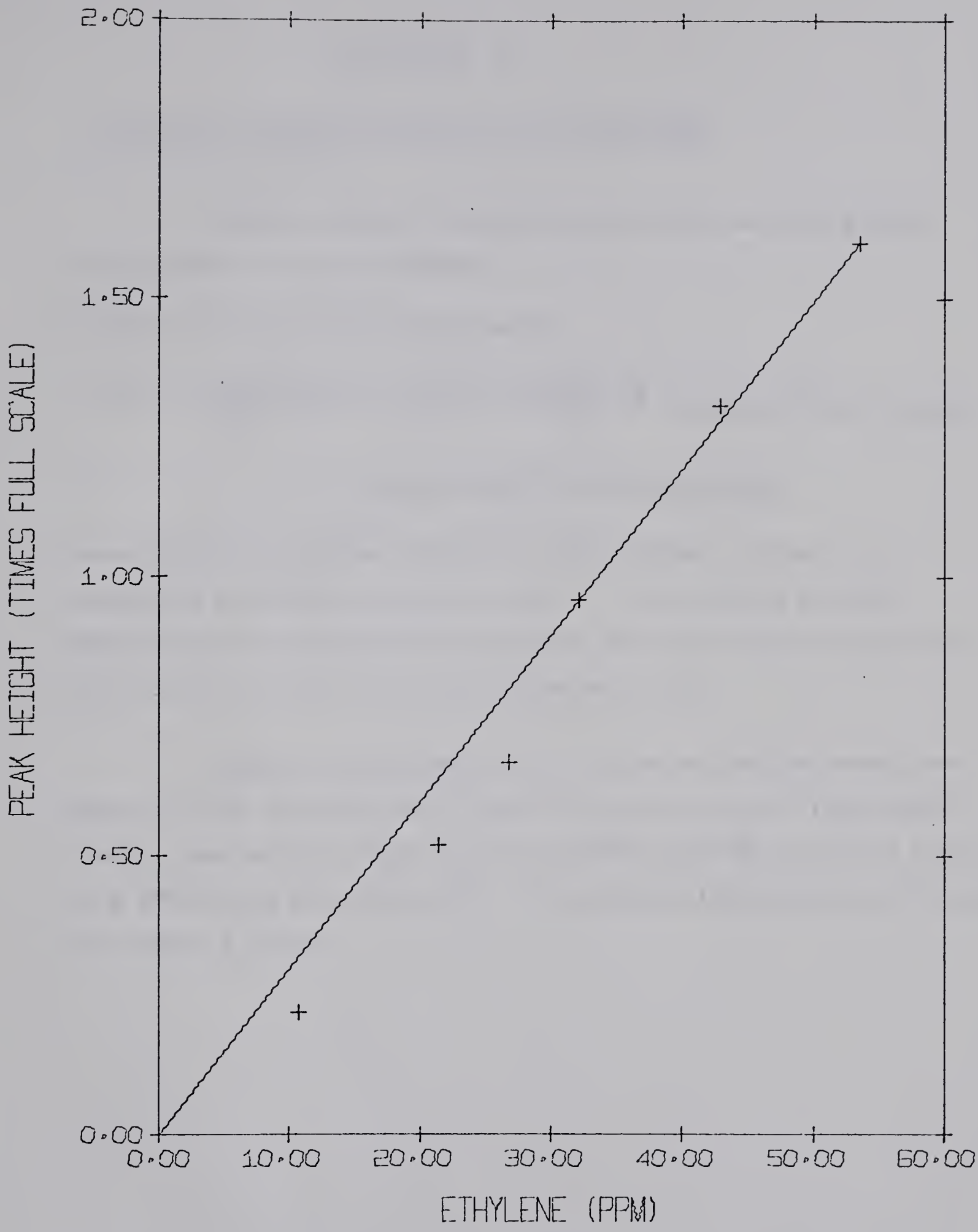
$= 0.0225$ μequiv OH^- or H^+

D. The Total H^+ Movements

The total change in amount of H^+ is equal to the sum of the results from parts B and C.

Total change = 0.000768 + 0.0225

= 0.023 μequiv H^+



APPENDIX B. STANDARD CURVE FOR GAS CHROMATOGRAPH RESPONSE TO ETHYLENE

APPENDIX C

OXYGEN CONCENTRATION CALCULATIONS

Oxygen uptake or release by cells was calculated from polarographic curves as follows:

O_2 change (μmoles $O_2/10^6$ cells per h)

$$= \frac{\text{slope (\% chart width)}}{\text{min}} \times \frac{60 \text{ min}}{1 \text{ h}} \times \frac{\mu\text{moles } O_2}{\text{ml}} \times \frac{1}{\text{number} \times 10^6 \text{ cells/ml}}$$

$$\times \frac{1}{\text{\% chart width at 100\% saturation}}$$

Photosynthetic O_2 uptake = apparent photosynthetic release O_2 + respiratory uptake for the same sample. For samples showing initial changes in the rate of respiration, the later slower respiratory rate was used to correct both photosynthetic rates.

Oxygen concentration of air saturated distilled water was taken to be 246 μmoles/l at 21° and 216 μmoles/l at 28° (calculated from O_2 concentration H_2O, 21° is 0.004252 gm/100 gm H_2O at 1 atm and 0.003718 gm/100 gms at 28°. Handbook of Chemistry and Physics 43rd edition p. 1706).

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